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(54) Title: PLANT ELONGATION FACTOR, PROMOTERS, CODING SEQUENCES AND USES

(57) Abstract

Expression constructs are provided employing a plant EF-1α promoter which allows for elevated expression in rapidly dividing cells. Sequences from the gene and untranslated regions associated with the gene may be employed in an antisense construct to reduce growth rate. The promoter finds particular use in protecting rapidly dividing tissue and tender shoots from a wide variety of environmentally induced stress conditions.

PLANT ELONGATION FACTOR, PROMOTERS,
CODING SEQUENCES AND USES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. application serial number 07/335,133, filed April 7, 1989, which is a continuation-in-part of U.S. application serial number 07/234,187, filed August 18, 1988, which disclosures are hereby incorporated by reference.

INTRODUCTION

Technical Field

The field of this invention relates to compositions and methods for modification of plant phenotype by preferential transcription of a gene of interest in specific tissue(s).

Background

With an increasing population, there is a continual demand for an increasing supply of food staples. In addition, in the wealthy societies, there is an expanding interest in food variety and improvement in organoleptic properties. Much of the revolution in agriculture in the increase of land utilization has been a result of the use of fertilizers. However, the use of fertilizers is a mixed blessing, in that the soil becomes ultimately less productive and the ground water becomes contaminated, resulting in eutrophy in various bodies of water.

With the ability to introduce DNA into plants, there is the potential to modify the phenotype of the plant. Various modifications have already been made and continue to be made. One modification of interest has been herbicide resistance. By providing for the crop being herbicide resistant, one can provide for clear discrimination between the crop and weeds. Thus, the weeds

may be destroyed, so that there is no diversion of nutrient and water to unproductive plants.

Initially, constitutive production of herbicide resistance was introduced into plants, so that there was a

5   substantially constant amount of the herbicide resistant product being produced throughout the plant, regardless of the utility of such capability in various tissues. A further improvement resulted in the modification in the herbicide resistant gene with a leader sequence which

10 allowed for transport of the gene into the chloroplast where the herbicide target was in the chloroplast. Since some herbicides inhibit enzymes present in the chloroplast providing for a herbicide-resistant enzyme in the chloroplast would maintain the enzyme function at the

15 natural site where it is utilized. Other improvements have involved introduction of insect resistance, where an insecticidal protein existing naturally in bacterial organisms is introduced into plants for expression and resistance of the plant to insects.

20   In many instances, there is a particular interest in protecting rapidly dividing cells or rapidly growing tissue against stress or other detrimental factors. For example, the site of action of various herbicides is rapidly dividing cells. Insects frequently target young tender

25 tissue for attack resulting in injury at this site. Certain plant diseases are particularly severe with young rapidly dividing cells. Also, the tender new tissue is most sensitive to stresses such as frost, so that enhanced production of products which protect against frost or

30 inhibition of endogenous products which enhance the sensitivity to frost is of primary interest in such tissue.

There is, therefore, substantial interest in being able to target particular products to rapidly dividing cells in young tissue. In some instances, the protein

35 which is produced may be deleterious to other tissue at elevated levels, so that one wishes to restrict expression

to the rapidly dividing cells. In other situations, the
product will have no utility at other sites, so that the
energy of the plant is wasted, reducing growth efficiency.
Toward this end, it is important to identify genes and
5   their promoters which will allow for expression in selected
tissue(s) at a desired high level, while providing for low-
or no-level of expression in other tissue.

Relevant Literature
10   Genes encoding elongation factor-1α from several
species have been cloned and sequenced. These include
yeast (Nagata, et al., EMBO J. (1984) 3:1825-1830); the
brine shrimp Artemia salina; (van Hemert, et al., FEBS
Lett. (1983) 157:295-299; the fungus Mucor racemosus (Linz,
15 et al., J. Biol. Chem. (1986) 261:15022-15029); and man
(Brands, et al., Eur. J. Biochem. (1986) 155:167-171). For
a review of elongation factor-1α (EF-1α) protein, see
Ejiri, Methods in Enzymology (1986), 118:140-153.

20   BRIEF DESCRIPTION OF THE DRAWINGS
        Figure 1 shows the sequence of LeEF-1.
        Figure 2 shows amino acid comparisons between tomato
and other EF-1α proteins. 1 = L. esculentum (tomato; LeEF-
1 this paper); 2 = A. salina (brine shrimp) (van Hemert et
25 al., 1984); 3 = H. sapien (man) (Brands et al., 1986); 4 =
M. racemosus (a dimorphic fungus) (Linz et al., 1985); 5 =
S. cerevisiae (yeast) (Nagata et al., 1984). Boxes
indicate residues which are homologous tomato EF-1α.
        Figure 3 shows the sequence of the EF-1α genomic
30 clone, 61-1 from tomato.
        Figure 4 shows the sequence of the EF-1α genomic
clone, clone A, also referred to as LeEF-A.
        Figure 5 is a graph representing a comparison of
expression levels of various constructs of a flurometric
35 GUS assay in transformed tobacco plants. Two EF-1α-GUS
constructs (pCGN2157 and pCGN2158) are represented and

compared to a mas-GUS construct (pCGN7001) and repeated
testing of a single double 35S-GUS clone (pCGN7320).

SUMMARY OF THE INVENTION

5   Novel DNA transcription constructs, together with
methods for their preparation and use, are provided for
modifying the phenotype of plant cells and tissues. Also
provided is a DNA sequence for preparation of the
transcription constructs. When transformed into a plant
10   cell, the transcription constructs provide for a
transcription pattern of a gene of interest associated with
that of elongation factor-1α (EF-1α). The DNA comprises
the 5' non-coding regions associated with the plant EF-1α
to and including the entire coding region for the plant EF-
15   1α and also the 3' non-coding region. DNA transcription
constructs are prepared which include the transcriptional
and translational regulatory region of EF-1α and a gene of
interest other than the EF-1α gene under the regulatory
central of the regulatory region and a termination region
20   functional in a plant cell. The transcriptional construct
can be used to insert any of a variety of endogenous or
exogenous genes into a plant cell for high level production
of transcription products, as well as translation products
associated with varying the phenotype of specific plant
25   cells and tissue.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel DNA sequences, constructs employing such
sequences, plant cells containing such constructs, and
30   plants containing such constructs, are provided, where the
sequences are associated with elongation factor-1α (EF-1α).
In addition, a method is described for identifying the
genes for EF-1α, since it is found that EF-1α is a multi-
gene family in plants. Plant EF-1α is characterized by
35   having a mRNA of about 1.5-2 kb, a molecular weight in the
range of about 45-55 kd, and a PI of about 8.5 to 9.5. The

polypeptides encoded by plant EF-1αs have substantial homology with the polypeptides encoded by EF-1αs from a wide variety of species.

The transcriptional initiation regulatory region is
5   found to provide regulation, where enhanced levels of EF-1α are expressed in rapidly dividing tissue, while lower levels are expressed in mature cells and tissue. An EF-1α gene isolated may be used to regulate expression in transgenic plants. For example, a promoter derived from an
10   EF-1α gene has been shown to direct expression of a foreign gene in tomato and in tobacco plants.

Significant levels of plant EF-1α mRNA are found in substantially all tissues, e.g. young leaf, young root, mature leaf, mature root, green fruit, turning fruit and
15   ripe fruit, generally ranging in values from 0.1 to 1.0%, as evidenced with the experience in tomato. Levels of EF-1α mRNA decrease in both the leaves and roots as they mature, and in fruit as it ripens.

The plant EF-1α sequence may be isolated from any
20   convenient plant. When used in a construct, the EF-1α sequence may be endogenous to the target host or exogenous to the target host. Plants from which EF-1α may be isolated include fruit, vegetables, oil seeds, fiber sources, grasses, trees, flowers, grain, ornamentals and
25   the like. Particular plants of interest include tomato, pea, tobacco, maize, soybean, Brassica, cotton, wheat, alfalfa, turf grass, and the like.

The plant EF-1α gene may be isolated by various techniques. These include isolating mRNA from a host
30   organism which codes for the EF-1α, reverse transcribing the mRNA, and using the resulting single-stranded (ss) DNA as a template to prepare double-stranded (ds) DNA and the ds DNA isolated. Among multi-gene families it is desirable to find the transcriptional initiation regulatory region
35   which provides a high level of transcription. Thus, the transcriptional initiation regulatory region should provide

for at least about 10% of the total EF-1α mRNA, preferably at least about 20%, and more preferably at least about 30%. This can be determined by employing two probes, one probe which is conserved and binds to all EF-1α mRNA, and the
5   other probe being in a polymorphic region of the EF-1α locus which binds uniquely to the EF-1α gene being assayed.

The sequences provided in the experimental section may be used to identify plant EF-1α genes from plant sources other than tomato. Particularly, by identifying sequences
10 of the subject plant associated with the EF-1α gene, which sequences are conserved in species other than plants, these conserved sequences may be used as probes for hybridization to cDNA obtained from a number of different plant sources. Usually, the sequence will have at least about 60%,
15 preferably at least about 70%, identity of base pairs, excluding any deletions which may be present. Thus cDNA libraries may be prepared from the plant source of interest, and the probes used to identify cDNA sequences for EF-1α. Conveniently, the target cDNA may be cloned in
20 a virus, so that hybridizing phage may be plaque-purified. The identified cDNA's may be further sub-cloned and the sub-clone sequence analyzed and used for production of probes. The probes are then used to identify cDNA EF-1α sequences in a cDNA library. The cDNA is used to identify
25 genomic sequences in a plant genomic library of the appropriate plant species and the positive clones analyzed by restriction enzyme digestion. The level of transcription may then be determined in a variety of plant tissues to demonstrate the pattern of transcription in the
30 plant. In this manner, one or more sequences may be identified providing both the coding region, as well as the transcriptional regulatory elements of the gene.

The probes can be considerably shorter than the entire sequence, but should be at least about 10, preferably at
35 least about 15, more preferably at least about 20 nucleotides in length. Longer oligonucleotides are also

useful, up to the full length of the gene encoding the
polypeptide of interest. Both DNA and RNA probes can be
used.

5   In use, the probes are typically labeled in a
detectable manner (for example with ^{32}P-labelled or
biotinylated nucleotides) and are incubated with single-
stranded DNA or RNA from the organism in which a gene is
being sought. Hybridization is detected by means of the
label after single-stranded and double-stranded

10   (hybridized) DNA or DNA/RNA have been separated, typically
using nitrocellulose paper. Hybridization techniques
suitable for use with oligonucleotides are well known to
those skilled in the art.

Although probes are normally used with a detectable

15   label that allows for easy identification, unlabeled
oligonucleotides are also useful, both as precursors of
labeled probes and for use in methods that provide for
direct detection of DNA or DNA/RNA. Accordingly, the term
"oligonucleotide" refers to both labeled and unlabeled

20   forms.

Once the 5'- and 3'-non-coding regions of the EF-1α
gene have been identified, they may be manipulated in
accordance with conventional ways. Where a convenient
restriction site is present downstream from the ATG

25   initiation codon, it may be useful to cut at that site and
insert a DNA sequence of interest for transcription under
the regulatory central of the EF-1α regulatory elements.
Where the DNA sequence of interest is a structural gene,
the sequence of interest will be inserted so as to be in

30   reading frame with the upstream codons and the initiation
methionine. Where the DNA is to be used as an anti-sense
sequence, it need not be in reading frame with the upstream
codons.

Usually, not more than 20 codons at the 5'-end of the

35   EF-1α gene will be retained. Preferably, the sequence of
interest will not be fused to the 5'-region of the EF-1α

gene but rather may be joined to the transcriptional
initiation regulatory region of EF-1α in a variety of ways.
One can cleave internal to the EF-1α gene and resect using
Bal31. By blunt-end ligation of the gene of interest to

5 the various EF-1α fragments, one can screen for expression
of the gene of interest indicating that a functional
transcription initiation region has been retained. To join
a sequence, one may use the polymerase chain reaction where
an oligonucleotide containing the sequence of interest

10 hybridizes at one end with the terminal sequence of the EF-
1α transcriptional initiation regulatory region and/or 5'-
untranslated region. By providing for replication using
the polymerase chain reaction, dsDNA may be prepared for
insertion into a vector which will have the EF-1α

15 transcriptional initiation regulatory region properly
joined to all of, or the 5'-proximal portion of, the
sequence of interest.

Conveniently, one may identify a convenient
restriction site in the 5'-untranslated region of EF-1α and

20 in the 5' region of the gene of interest and employ an
adapter which will join the two sequences and restore the
lost sequences from the 5'-untranslated region and the 5'
region of the gene of interest. Alternatively, one may
introduce a polylinker immediately downstream from the 5'-

25 untranslated region, for insertion of the DNA sequence of
interest.

The 5'-non-coding region which will be employed for
joining to the sequence of interest will usually be at
least about 100 bp, and not more than about 10 kbp,

30 frequently being less than about 2kbp, and may include all
or a portion of an intron (including the splice sites).
The 5'-non-coding region which is employed will be proximal
to (usually within 20 bp) or abut the initiation codon.
Therefore, by employing sequence analysis, one can identify

35 the initiation codon of the EF-1α gene and isolate the
upstream region in accordance with conventional ways.

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Where a particular cloned fragment in a genomic library
does not have the desired size, the library may be further
screened by walking the 5'-non-coding region until a
fragment of the desired length is obtained. The 5'-non-
5 coding region may then be cloned and sequenced and may then
be used for further manipulation. As already indicated, in
some instances, at least a fragment of the 5'-coding region
may be retained.

The EF-1α transcriptional initiation regulatory region
10 (promoter) may be inserted into a vector for preparing a
construct for transcription and optionally translation.
The 3' terminus of the EF-1α promoter may be joined to a
sequence of interest, which may be a structural gene or
coding or noncoding sequence where anti-sense may be
15 desired. A wide variety of genes are of interest for
transcription and optionally translation in rapidly
dividing cells. For example, tender shoots and tissue are
particularly sensitive to stress. Stress may take the form
of toxins, such as pesticides, or herbicides, temperature
20 changes such as heat or cold, including ice formation,
osmotic pressure as a result of salinity, drought, and the
like. Stress may include attack by various organisms or
susceptibility to disease, as evidenced by insects,
nematodes, aphids, caterpillars, fungi, and the like.
25 Growth may be modulated, either increased or decreased,
depending upon the particular need. Anti-sense sequences
may be used to reduce growth, for example, altering the
auxin/cytokinin ratio may be used to alter growth rate
and/or morphology. By enhancing or diminishing the
30 expression of an enzyme in the metabolic pathway for the
hormone, the ratio may be modulated.

For resistance to toxins, various genes may be
employed which may be the target of the toxin, where a
mutated gene is employed which is less sensitive or
35 insensitive to the toxin. Alternatively, one can enhance
the amount of the target protein, so as to reduce the

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sensitivity of the plant cells to the toxin. For example,
5-enolpyruvyl-2-phosphoshikimate synthase is the target for
glyphosate (aroA gene). By providing for an aroA gene
which is less sensitive to the herbicide and/or enhancing
5   the level of the enzyme present in the cell, particularly
in the chloroplast, herbicide-resistance may be achieved.
Alternatively, a gene which provides for detoxification may
be employed, such as a nitrilase which hydrolyzes the
nitrile group of bromoxynil. Additional examples are the
10 herbicide phosphinothricin which may be detoxified by
phosphinothricin acetyltransferase from Streptomyces
hygroscopicus (bargene) and acetolactate synthase which may
be mutated to be resistant to various sulfanyl-urea
herbicides.

15   For insects or nematodes, there are naturally
occurring toxins for the pest which may be provided as a
gene for expression in the plant cells. For example a
number of strains of Bacillus thuringiensis have relatively
·broad spectrum toxicity to a number of different insects as
20 a result of expression of a protein toxin. By providing
for expression of the protein toxin in plant cells,
particularly among rapidly dividing cells, these cells will
be protected from attack by such insects. Protection may
also be provided against nematodes and fungus, and root
25 worm, e.g. corn root worms.

For providing resistance to frost or heat, various
genes may be employed, such as heat shock proteins, winter
flounder antifreeze protein, etc. For stress tolerance
such as salinity, Osm genes may be employed, such as genes
30 associated with overproduction of proline. Additionally,
stress tolerance may be provided by genes for superoxide
dismutase.

The auxin/cytokinin ratio may be modified by
modulating genes on the metabolic pathway to protect
35 growing cells or to change growth rate. Other DNA

sequences of interest in this regard include antisense EF-1α which may be used to modulate expression of EF-1α.

The EF-1α promoter, being a strong promoter, may be used in place of other strong promoters conventionally

5 employed, such as the 35S cauliflower mosaic virus promoter. The subject promoters may also find use for a specific selectable marker where the target for transformation and regeneration is merisystematic tissue e.g., for use with particle bombardment in cotton, soybean,

10 etc.

Depending upon the sequence of interest, the purpose of the transformation and the particular host, other sequences may be included which provide for specific functions. In some instances, it may be desirable to

15 provide for translocation of the expression product from the cytoplasm to an organelle or for secretion. In this instance, various transit peptides may be employed for translocating the gene of interest to an organelle, such as the chloroplast or mitochondrion, or to secrete the protein

20 into the extracellular space or to the cell surface. Various transit peptides have been employed, such as the transit peptide of the small subunit of the RUBISCO gene, plant EPSP synthase, acyl carrier protein, and the like.

In addition to the promoter and the structural gene,

25 there will be a termination region, which may be the EF-1α termination region or any other convenient termination region. A wide variety of termination regions have been employed, such as termination regions from opine genes, various plant genes, and the like. The particular

30 termination region will usually not be critical to this invention and any convenient region may be employed.

The promoter, structural gene and transcriptional and translational termination region provide an expression construct which may be joined to a vector for cloning. At

35 each stage of the preparation of the construct, the resulting product may be cloned and analyzed to insure that

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the desired product has been obtained. Cloning vectors
conveniently will have one or more markers, which will
allow for detection of transformants which contain the
construct and marker. For the most part markers will

5   provide for toxin resistance or impart prototrophy to an
auxotrophic host. Toxin resistance for the most part will
be antibiotic resistance, such as resistance to kanamycin
and its analogs, e.g. G418, resistance to chloramphenicol,
and the like. For the most part cloning will be performed

10 in E. coli, so that a replication system functional in E.
coli will be employed.

    Once the expression construct has been prepared and
analyzed to insure it has the proper sequence, it may be
then be used for introduction into a plant cell. Various

15 techniques exist for introduction of DNA into plant cells.
These techniques include A. tumefaciens mediated
introduction, electroporation, protoplast fusion,
injection, high velocity projectile introduction, and the
like. The targets for introduction of the DNA may be

20 tissue, particularly leaf tissue with A. tumefaciens,
disseminated cells, protoplast, seeds, embryo, meristematic
regions, cotyledons, hypocotyls, and the like.

    With A. tumefaciens introduction, the construct will
be further modified by having one or both T-DNA borders

25 present, bordering the expression construct, particularly
the right border. The construct may be introduced into A.
tumefaciens carrying the vir genes, where the T-DNA
bordered expression construct will be introduced into plant
cells infected with A. tumefaciens. The plants which may

30 be genetically modified include those plants described as
the source of the EF-1α gene. Thus, of particular interest
are those crops associated with food sources, such as
grain, vegetables and fruits, oil seed, sugar sources,
forage, and legumes.

35 Once the cells are transformed, transgenic cells may
be selected by means of a marker associated with the

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expression construct. The expression construct will
usually be joined with a marker which will allow for
selection of transformed plant cells, as against those
cells which are not transformed. The marker will usually
5   provide resistance to an antibiotic, which antibiotic is
toxic to plant cells at a moderate concentration.

After transformation, the plants cells may be grown in
an appropriate medium. In the case of protoplasts the cell
wall will be allowed to reform under appropriate osmotic
10 conditions. In the case of seeds or embryos, an
appropriate germination or callus initiation medium would
be employed. For explants, an appropriate regeneration
medium would be used.

The callus which results from cells may be introduced
15 into a nutrient medium which provides for the formation of
shoots and roots and the resulting plantlets planted and
allowed to grow to seed. During the growth, tissue may be
harvested and screened for the presence of expression of
the expression construct. After growth, the seed may be
20 collected and replanted, or prior to seed formation, the
modified plant may be used for fertilizing a different
strain or vice versa, so as to provide for a hybrid plant.
One or more generations may then be grown to establish that
the gene is inherited in Mendelian fashion.

25   Of particular interest is a gene associated with
tomato, which has the amino acid and DNA sequence found in
the Experimental section. The gene and its accompanying
untranslated regions may be used in the anti-sense
direction to inhibit expression in plant cells, so as to
30 reduce growth. Thus, fragments of the gene and the
untranslated regions of at least about 12 bp, usually at
least about 20 bp may be employed to produce an mRNA which
results in reduction in expression of the EF-1α gene.

The invention now being generally described, it will
35 be more readily understood by reference to the following
examples which are included for purposes of illustration

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only and are not intended to limit the invention unless so
stated.

EXPERIMENTAL

Example I

EF-1α cDNA

A.   Materials and Methods

    1.   Screening a Tomato cDNA Library for EF-1α Clones

        A red, ripe tomato (Lycopersicon esculentum cv
"Caligrande") cDNA library in λgt10 (Sheehy, et al., Mol.
Gen. Genet. (1987) 208:30-36) was screened for EF-1α cDNAs.
1 x 10^{5} recombinant plaques of the λgt10 library grown in
E. coli strain C600 hflA150 (Hoyt, et al., Cell (1982)
31:565-573) were transferred to GeneScreen Plus filters
(New England Nuclear) denatured, and neutralized by a
modification of the procedure of Benton and Davis, Science
(1977) 196:180-182, as described in O'Neal et al., Nucl.
Acids Res. (1987) 15:8661-8667). The probe used was
isolated from pMUEF-1 which contains the Mucor racemosa EF-
1α gene, TEF-1 (Linz, et al., Mol. Cell. Biol. (1986)
6:593-600. A 471 bp ClaI fragment from pMUEF1-2, which
spans nucleotides 219 to 689 of TEF-1 (corresponding to
amino acids 73 to 230) (Linz, et al., (1986) Mol. Cell.
Biol., 7:1925-1932) was isolated and nick-translated as
previously described (Shewmaker, et al., Virology (1985)
140:281-288). Prehybridization, hybridization and washing
of filters were done at a temperature of 33°C.

        Ten separate plaques corresponding to ten different
positive signals were picked, replated, and rehybridized
until all were plaque purified. The inserts on these ten
clones were sized by EcoRI digestion. A clone with an
approximately 1.7 kb insert, designated LeEF-1, was chosen
for further analysis. The EcoRI fragment of LeEF-1 was
subcloned into pUC19 (Yanisch-Perron et al., Gene (1985)
33:103-119) yielding pCGN666. This EcoRI fragment was also
cloned in both orientations into M13mp19 yielding pCGN667
and pCGN668, and into Bluescript M13+KS (Stratagene),

yielding pCGN669 and pCGN670. These subclones were used
for sequence analysis and generation of ^{35}S-RNA probes.
The cDNA sequence of pCGN666 has been filed with the EMBO
Gene Bank Data Library Postfach 10.2209, Meyerhofstrasse 1,
5   6900 Heidelberg, West Germany, on March 6, 1989. The file
number is X14449.

Sequencing Analysis

The cDNA in pCGN670 was sequenced in the 5' to 3'
10 direction using ExoIII-S1 nuclease nested deletions (SacI,
BamHI double digested) (these deletions were made using the
Promega Biotec Erase A-Base kit according to manufacturer's
instructions). The dideoxy method (Sanger, et al., Proc.
Natl. Acad. Sci. USA (1977) 74:5463-5467 was used to
15 sequence subclones from the deletion series. To overcome
gaps not covered by the nested deletions, oligomers were
synthesized (Applied Biosystems 380 A synthesizer, Foster
City, CA) based on known sequences from the previously
sequenced subclones. The oligomers served as primers for
20 the pCGN667 template. For sequence confirmation, the EF-1α
cDNA was also sequenced in the 3' to 5' direction using the
Sanger method on pCGN668 utilizing synthetic oligomer
primers. To sequence the region nearest the polyA$^+$ tail in
pCGN668, the Maxam-Gilbert method (Maxam and Gilbert, Proc.
25 Natl. Acad. Sci. USA (1977) 74:560-564) was used,
sequencing through the polyA$^+$ tail and a small distance
beyond, to overlap with the previously obtained sequence.

Isolation of RNA and Northern Analysis

30 Tissue from various anatomical parts and developmental
stages of tomato cultivar UC82B was frozen in liquid
nitrogen until needed for analysis. From mature three and
a half month old green house grown tomato plants (UC82B),
the following tissue was taken: mature leaf, mature root,
35 young leaf, green fruit, turning fruit, and red fruit
(fruit ripening stages were determined from Color

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Classification Requirements in Tomato, USDA Visual Aid TM-
:1; Feb. 1975, The John Henry Co., P.O. Box 17099, Lansing,
MI). The entire compound leaf (stem included with
leaflets) was harvested to isolate mature leaf RNA. Mature
5   root RNA was isolated from the cleaned-up root ball. Young
leaves were collected as axillary shoots (about five inches
from base to top). Tissue from young root was collected
from one and one-half week old germinated seedlings of
UC82B. Seeds were surface sterilized in a 0.1% sodium
10   hypochlorite, 0.1% Tween 20 (Sigma Chemical Co.) solution
for 15 minutes, rinsed in sterile, distilled water and
germinated on moistened autoclaved germinating paper
(Anchor Paper Co., St. Paul, MN).

        Tomato total RNA was prepared as described in Colbert,
15   et al., Proc. Natl. Acad. Sci. USA (1983) 80:2248-2252 with
a slight modification (Facciotti, et al., Bio/Technology
(1985) 3:241-246). PolyA+ mRNA was isolated by oligodT
cellulose column chromatography as described in Colbert, et
al. (1983) supra; Maniatis, et al., Molecular Cloning: A
20   Laboratory Manual, Cold Spring Harbor, New York (1982).

        Northern analysis using nick-translated probes was
performed as described (Facciotti, et al., supra). When
olignoucleotides were used as probes for Northern analysis,
the procedure was performed as described in O'Neal, et al.,
25   Nucl. Acids Res. (1987) 15:8661-8667, except that the final
washes were with 0.5xSSC, 0.1% SDS at 45°C, for 2 x 15 min.

DNA Isolation and Southern Analysis

        Bacteriophage DNA was isolated from crude cell lysates
30   with the Lambda Sorb (Promega Biotech) phage absorbent
using the manufactuer's protocol. Tomato genomic DNA was
isolated by the CTAB (cetyltrimethlammonium bromide)
procedure (Taylor and Powell, Focus (1982) 4:4-6).
        Southern analysis was performed as described in Shewmaker,
35   et al., (1985), supra.

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In vitro translation analysis

        Poly(A+) mRNA was isolated from the fruit of cultivar
"UC 82B" by the method of Sheehy, et al., Mol. Gene. Genet.
(1987) 208:30-36. Approximately 0.5 μg of polyA+ RNA was
5   translated in a reticulocyte lysate (Promega Biotech) as
described by the manufacturer. 35S-methionine-labelled
translation products were precipitated with five volumes of
acetone and collected by centrifugation. Samples were
subjected to nonequilibrium two-dimensional gel
10 electrophoresis as described by Garcia, et al., J. Bact.
(1980) 142:196-201.

35S-RNA Probe Generation

        Sense and antisense 35S-RNA probes were generated from
15 pCGN669 and pCGN670 respectively using T3 polymerase an an
in vitro transcription reaction. These plasmids were
linearized at a unique BamHI site within the Bluescript
polylinker. DNA was purified after BamHI digestion by
extracting with phenol twice and phenol/chloroform/isoamyl
20 alcohol (25:24:1) once. The extracted DNA was then ethanol
precipitated and was resuspended in sterile water at a
concentration of 2μg/μl. In an RNase-free microfuge tube,
the following were mixed in the order given: 2μl 5X
transcription buffer (200 mM Tris, pH7.5, 30 mM MgCl2, 10
25 mM spermidine), 0.5 μl 100 mM DTT, 0.5 μl RNase inhibitor
(RNasin) (25 U/μl, Promega Biotech), 2μl 10X rNTPs (2,5 mM
GTP, 2.5 mM ATP, 2.5 mM CTP, 75 μM UTP), 4.5 μl α-35S-UTP
(10 μCi/μl, New England Nuclear), 0.5 μl DNA (2 μg/μl), and
0.5 μl T3 polymerase (50 U/μl, Stratagene). After careful
30 mixing, the reaction mixture was incubated at 37°C for one
hour. After one hour, 0.5 μl RNasin (25 U/μl, Promega
Biotech) and 0.5 μl RQ1 DNAse (1 U/μl, Promega Biotech)
were added and the reaction incubated for an additional 10
minutes. The reaction was stopped by the addition of 1 μl
35 250 mM EDTA, pH 7.0. Unincorporated ribonucleotide
triphosphates were separated from RNA transcripts by

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Sephadex G-75 gel filtration chromotagraphy. Both pCGN669
and pCGN670 had labeled peaks of approximately 4.0 X 10^8
cpm/μg RNA.

5   In situ RNA Hybridization
       Root tips from one and one-half week old tomato
seedlings were fixed in a 4% paraformaldehyde/phosphate
buffered saline (PBS)/5mM MgCl2 solution, pH 7.4 (PBS is 10
mM pH 7.4 phosphate buffer, made to 150 mM NaCl) (Singer,
10   et al., Biotechdniques (1985) 4:230-250). Tissue was fixed
overnight. After fixation, the tissue was passed through a
graded tertiary butyl alcohol (TBA) series starting at 50%
TBA, infiltrated with Paraplast and cast into paraffin
blocks for sectioning, (Berlyn and Miksche (1976) Botancial
15   Microtechnique and Cytochemistry, State University Press,
Ames, Iowa). Embedded root tips were sectioned
longitudinally on a Reichert Histostat rotary microtome at
4 μM to achieve a one-cell layer thickness. Paraffin
ribbons holding 7-10 root tip sections were affixed to
20   gelatin-chrom alum subbed slides (Berlyn and Miksche
(1976), supra) and held in a dust-free box until in situ
hybridizations were performed. Slides ready to be
hybridized were deparaffinized in xylene and rehydrated by
passing through an ethanol hydration series as described in
25   Singer, et al., Biotechniques (1986) 4:230-250.
       A 2X hybridization mix was made consisting of 100 μl
20X SSC, 20 μl 10% BSA, 100 μl 750 mM DDT, 200 μl 50%
dextran sulfate, 50 μl RNasin, and 30 μl sterile water.
2.5 μl tRNA (20 mg/ml), 2.5 μl salmon sperm DNA (10 mg per
30   ml) and 4 X 10^6 cpm probe were dried down on a lyophilizer.
This mix was then resuspended in 25 μl 90°C formamide and
25 μl 2X hybridization mix per slide. 40 μl of this
hybridization mix was placed on each slide. A cover slip
was placed over the sections and edges sealed with rubber
35   cement. Slides were placed in slide holders inside a glass
slide box, covered, and placed in a 37°C dry oven overnight

to hybridize. Posthybridization treatments were as described in Singer, et al., (1986), supra.

Autoradiography was performed as described in Kodak Materials for Light Microscope (1986) (available from

5 Kodak), using Kodak liquid emulsion NTB-3. Slides were exposed in a light box for approximately two weeks. After developing the autoradiographic slides, root sections were stained in 0.05% toluidine blue and then dehydrated through a graded alcohol series: xylene: 100% ethanol, 1:1,

10 followed by 2 changes 100% xylene. Slides were left for five minutes in each solution. Coverslips were mounted with cytoseal (VWR) and left on a slide warmer until dry (45-50°C, 1-2 days). Autoradiographic slides were then ready for microscopic examination and photomicrography.

15

B. Results

To isolate a tomato EF-1α cDNA clone, a fragment from a Mucor racemosa EF-1α cDNA clone was used (Linz and Sypherd, Mol. Cell Biol. (1987) 7:1925-1932). Preliminary

20 analysis using this probe to tomato RNA indicated that under the conditions used, the Mucor probe hybridized solely to a bind of approximately 1.8kb. This agrees with the size reported for other EF-1α cDNA RNA's (Nagata, et al., EMBO J. (1984) 3:1825-1830; Linz et al., Mol. Cell

25 Biol. (1986) 6:593-600), and is within the expected size range of the protein (45-55kd).

The clone containing the largest insert of 1.7kb, hereinafter termed LeEF-1, was chosen for further analysis.

LeEF-1 contains 60 nucleotides upstream of the ATG, a

30 1344 nucleotide (448 amino acids) coding region and 284 nucleotides of 3'-untranslated region. In the 5'-untranslated region there are two in-frame stop codons. In-frame stop codons are also found in the 5' untranslated regions of other EF-1α genes. In the LeEF-1, an adenine

35 residue is at the -3 position and a guanine residue is at the +4 position relative to the start AUG codon. This

nucleic acid sequence surrounding the initiator codon
displays the consensus signal for efficient translation
initiation.   The complete sequence of LeEF-1 is shown in
Figure 1.

5   Amino acid sequence comparisons between the
polypeptides encoded by tomato EF-1α gene, LeEF-1 and an
EF-1α gene from Mucor, as well as Artemia, yeast, and man
show substantial homology.   The LeEF-1 gene product is 76%
homologous to that of yeast and Mucor; 75% homologous to

10   that of Artemia; and 78% homologous to that of man.

        As shown in Figure 2, one notable difference between
LeEF-1 and the four other eukaryotic EF-1α's is that a 12
amino acid gap must be introduced between amino acids 213
and 214 of the protein encoded by LeEF-1 to provide maximum

15   homology to the other eukaryotic EF-1α gene products.   The
homology between the polypeptides encoded by the four other
eukaryotic EF-1α genes in this gap region ranges from 29%
to 64% and no specific function has been previously
assigned to this region.

20

EF-1α Is A Multigene Family In Tomato

        To determine if EF-1α in tomato was encoded by one or
multiple genes, Southern blot analysis was performed.   When
tomato DNA restricted with EcoRI was hybridized to LeEF-1,

25   at least six distinct bands were seen.   This indicated that
EF-1α is probably a multigene family.   This was verified by
screening a tomato genomic library for EF-1α clones.   Six
distinct clones obtained from this screen were restricted
with EcoRI and hybridized to LeEF-1.   These six distinct

30   clones represent four of the bands seen on the genomic
Southern blot.   The EcoRI fragment from one of these clones
(61-1) was subcloned and shown to contain an entire EF-1α
gene (see Example II).   While it is clear that EF-1α in
tomato is a multigene family, the exact size of the family

35   remains to be determined.

EF-1α Is Expressed In Rapidly Growing Tissue

Various tomato mRNA's were run on a Northern gel and
hybridized against the tomato EF-1α cDNA. Specifically,
the probe was the 1.7 kb EcoRI fragment from pCGN666 which
5   had been labelled by nick-translation. Tissues chosen for
analysis were young leaf, young root, mature leaf, mature
root, green tomato fruit, turning tomato fruit, and red
ripe tomato fruit. Significant levels of EF-1α mRNA were
found in all tissues examined with values ranging from 0.1
10 to 1.0% of total mRNA. Levels of EF-1α mRNA decreased in
both the leaves and roots as they matured and in the fruit
as it ripened.

The expression pattern of EF-1α was further examined
in vitro translation. Poly(A$^+$) RNA from green and red
15 (mature) tomato fruit was isolated, translated and
subjected to 2-D gen analysis. The EF-1α protein is easily
identifiable because of its molecular weight (50,000
daltons) and its high pI of around 9. While EF-1α protein
is produced from mRNA of both green and red fruit, more is
20 produced by the RNA from younger green fruit. These data,
taken together with the Northern analysis, indicate that
EF-1α is a highly expressed protein in tomato and that this
expression is greater in young, rapidly growing tissue than
in older, more mature tissue.

25

5.   Localization of EF-1α RNA To Meristematic Regions of
The Root Tip

To further characterize the expression pattern of EF-
1α, in situ hybridizations were performed. In root tips
30 there are well-defined meristematic regions that are
associated with active cell division as well as regions
associated with cell elongation (Raven, et al., Biology of
Plants (1976); Essau, Anatomy of Seed Plants (1977);
Galston, et al., The Life of The Green Plant (1980)). When
35 4-mm root tips from young tomato plants were hybridized to
sense and antisense ^{35}S-EF-1α RNA, the antisense

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transcripts hybridized to distinct regions of the root
tips, while the sense transcripts (negative control) showed
no hybridization. More hybridization was seen in the first
1 to 2 mm of the root tip, which corresponds to the region

5   of cell division, than in the tissue further up the root
tip, known as the region of cell elongation. Strong,
defined hybridization was seen in the three primary
meristems: protoderm, ground meristem, and procambrium, as
well as in the apical meristem. The three meristems in a

10   root give rise to the epidermis, cortex and primary
vascular tissue respectively. Thus, within a root tip, the
highest levels of EF-1α mRNA were found in meristematic or
rapidly dividing cells. In summation, these _in situ_
results corroborated the Northern data to demonstrate that

15   EF-1α mRNA in tomato is particularly abundant in actively
dividing cells.

                         Example II
             ISOLATION AND CHARACTERIZATION OF AN
20   EF-1α GENOMIC CLONE 61-1

     A.   Materials and Methods
           1.   Screening a tomato genomic library for EF-1α
     genomic clones.

                 A tomato (Lycopersicon esculentum cv 'T6')
25   partial EcoRI genomic library was provided by Dr. Robert
     Goldberg, UCLA. This library was screened for EF-1α genes
     using the nick-translated tomato cDNA, LeEF-1 as a probe.
     Approximately eight tomato genome equivalents in 800,000
     plaques of the Charon4 phage in E. coli host LE392 (F⁻

30   hsdR514 supE44 supF58 lacY1 or lac(1-Y6 galK2 galT22 metB1
     trpR55 9λ⁻) were screened. DNA was transferred to Gene
     Screen Plus filters as described in Example I for screening
     the tomato cDNA. Prehybridization, hybridization, and
     washing of filters were as described in Shewmaker, et al.

35   .(1985), supra). Filters were prehybridized and hybridized
     at 42°C and washed at 55°C. A number of positive plaques

were picked and replated until plaque purified. The tomato inserts in positive clones were sized by EcoRI digestion.

   2.   Isolation, mapping and sequencing of an EF-1α genomic clone (61-1) from tomato.

       a.   Construction of pCGN2123

           One EF-1α lambda clone (61-1) containing a 5.6 kb EcoRI fragment that hybridized to LeEF-1 was chosen for further analysis. DNA from 61-1 was prepared using a Lambda Sorb kit (Promega Biotech) according to manufacturer's instructions. The 5.6 kb EcoRI piece was subcloned into pUC19 yielding pCGN2123. Using standard mapping techniques (Maniatis, et al., (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, N.Y.), the approximately 2 kb EF-1α gene was located within the 5.6 kb fragment. The coding region plus approximately 800 bp 5' of the start codon and approximately 1200 bp 3' of the stop codon were sequenced. Sequence analysis was performed using standard techniques, similar to those described for the sequencing of LeEF-1.

   3.   Construction of Binary Plasmid pCGN783

           pCGN783 is a binary plasmid containing the left and right T-DNA borders of A. tumefaciens (Currier and Nester, J. Bacterial, (1976), 126:157-165); the gentamicin resistance gene of pPH1JI (Hirsch, et al., Plasmid (1984) 12:139-141); the 35S promoter of cauliflower mosaic virus (CaMV) (Gardner, et al., Nucleic Acids Res. (1981) 9:2871-2888); the kanamycin resistance gene of Tn5 (Jorgenson et al., Mol. Gen. Genet., (1979) 177:65-); and the 3' region from transcript 7 of pTiA6 (Currier and Nester (1976), supra). pCGN783 was constructed by the multistep process described below. pCGN783 has been deposited with the American Type Culture Collection (ATCC 67868).

24

Construction of pCGN451

pCGN451 contains the ocs5'-ocs3' cassette cloned into
a derivative of pUC8 (Vieira and Messing, Gene, (1982),
5   19:259-268). The modified vector was derived by digesting
pUC8 with HincII and ligating in the presence synthetic
linker DNA, creating pCGN416, and then deleting the EcoRI
site of pCGN416 by EcoRI digestion followed by treatment
with Klenow enzyme and self ligation to create pCGN426.

10   The ocs5'-ocs3' cassette was created by a series of
steps from DNA derived from the octapine Ti-plasmid pTiA6
(Currier and Nester, J. Bact. (1976) 126:157-165). An
EcoRI fragment of pTiA6 (bp 13362-16202; the numbering is
by Barker, et al., Plant mol. Biol. (1983) 2:335-350), for
15   the closely related Ti plasmid pTi15955) was removed from
pVK232 (Knauf and Nester, Plasmid (1982) 8:45) by EcoRI
digestion and cloned into EcoRI digested pACYC184 (Chang
and Cohen, J.Bacteriol. (1978) 134:1141-1156) to generate
pCGN15.

20   The 2.4 kb BamHI-EcoRI fragment (bp 13774-16202) of
pCGN15 was cloned into EcoRI-BamHI digested pBR322
(Bolivar, et al., Gene (1977) 2:95-113) to yield pCGN429.
The 412 bp EcoRI-BamHI fragment (bp13362-13774) of pCGN15
was cloned into EcoRI-BamHI digested pBR3322 (Bolivar, et
25   al., Gene (1977) 2:95-113) to yield pCGN407. The cut-down
promoter fragment was obtained by digesting pCGN407 with
XmnI (bp 13512), followed by resection with Bal31
exonuclease, ligation of synthetic EcoRI linkers, and
digestion with BamHI. Resulting fragments of approximately
30   130 bp were gel purified and cloned into M13mp9 (Vieira and
Messing, Gene (1982) 19:259-268 and sequenced. A clone, I-
4, in which the EcoRI linker had been inserted at bp 13642
between the transcription initiation point and the
translation initiation codon was identified by comparison
35   with the sequence of de Greve et al (de Greve, et al., J.
Mol. Appl. Genet. (1982) 1:499-512). The EcoRI cleavage

site was at position 13639, downstream from the mRNA start
site. The 141 by EcoRI-BamHI fragment of I-4, containing
the cut-down promoter, was cloned into EcoRI-BamHI digested
pBR322 (Bolivar, et al., Gene (1977) 2:95-113) to create

5   pCGN428. The 141 bp EcoRI-BamHI promoter piece from
pCGN428, and the 2.5 kb EcoRI-BamHI ocs 5' piece from
pCGN429 were cloned together into EcoRI digested pUC9
(Vieira and Messing, Gene (1982) 19:259-268) to generate
pCGN442, reconstructing the ocs upstream region with a cut-

10 down promoter section.
        The HindIII fragment of pLB41 (D. Figurski) containing
the gentamicin resistance gene was cloned into HindIII
digested pACYC184 (Chang and Cohen, J. Bacteriol. (1978)
134:1141-1156) to create pCGN413b. The 4.7 kb BamHI

15 fragment of pTiA6 (Currier and Nester, J. Bact. (1976)
126:157-165) containing the ocs 3' region, was cloned into
BamHI digested pBR325 (F. bolivar, Gene (1978) 4:121-136)
to create 33c-19. The SmaI site a position 11207 of 33c-19
was converted to an XhoI site using a synthetic XhoI

20 linker, generating pCGN401.2. The 3.8 kb BamHI-EcoRI
fragment of pCGN401.2 was cloned into BamHI-EcoRI digested
pCGN413b to create pCGN419.
        The ocs5'-ocs3' cassette was generated by cloning the
2.64 kb EcoRI fragment of pCGN442, containing the 5'

25 region, into EcoRI digested pCGN419 to create pCGN446. The
3.1 kb XhoI fragment of pCGN446, having the ocs 5' region
(bp 13639-15208) and ocs 3' region (bp 11207-12823), was
cloned into the XhoI site of pCGN426 to create pCGN451.

30 Construction of pCGN587
        pCGN587 was prepared as follows: The HindIII-SmaI
fragment of Tn5 containing the entire structural gene for
APH3'II (Jorgensen, et al., Mol. Gen. Genet. (1979) 177:65-
) was cloned into HindIII-SmaI digested pUC8 (Vieira and

35 Messing, Gene (1982) 12:259-268). This converted the
fragment into a HindIII-EcoRI fragment, since there was an

EcoRI site immediately adjacent to the SmaI site. The
PstI-EcoRI fragment of pCGN300, containing the 3'-portion
of the APH3'II gene, was then combined with an EcoRI-BamHI-
SalI-PstI linker into the EcoRI site of pUC7 to make

5   pCGN546W. An ATG codon was upstream from and out of
reading frame with the ATG initiation codon of APH3'II.
The undersired ATG was avoided by inserting a Sau3A-PstI
fragment from the 5'-end of APH3'II, which fragment lacks
the superfluous ATG, into the BamHI-PstI site of pCGN546W

10   to provide plasmid pCGN550. The EcoRI fragment of pCGN550
containing the APH3'II gene was then cloned into the EcoRI
site of pUC8-pUC13-cm (K. Buckley, Ph.D. Thesis, UC San
Diego, (1985)) to give pCGN551. The plasmid pCGN451 having
the ocs 5' and ocs 3' in the proper orientation was

15   digested with EcoRI and the EcoRI fragment from pCGN551
containing the intact kanamycin resistance gene inserted
into the EcoRI site to provide pCGN552 having the kanamycin
resistance gene in the proper orientation. This ocs/KAN
gene was used to provide a selectable marker for the trans

20   type binary vector pCGN587.
       The 5' portion of the engineered octopine synthase
promoter cassette consisted of pTiA6 DNA from the XhoI site
at bp 15208-13644 (Barker, et al., Plant Mol. Biol. (1983)
2:335-350), and also contains the T-DNA boundary sequence

25   (border) implicated in T-DNA transfer. In the plasmid
pCGN587, the ocs/KAN gene from pCGN552 provided a
selectable marker as well as the right border. The left
boundary region was first cloned in M13mp9 as a HindIII-
SmaI piece (pCGN502) base pairs 602-2212 and recloned as a

30   KpnI-EcoRI fragment in pCGN565 (a cloning vector containing
vector based upon pUC8-cm but containing pUC18 linkers) to
provide pCGN580. pCGN565 is a cloning vector based on a
pUC8-pUC13-cm (K. Buckley (1985), supra) but containing
pUC18 linkers (Yanisch-Perron, et al., Gene (1985) 33:103-

35   119) pCGN580 was linearized with BamHI and used to replace
the smaller BglII fragment of pVCK102 (Knauf and Nester,

Plasmid (1982) 8:45), creating pCGN585. By replacing the smaller SalI fragment of pCGN585 with the XhoI fragment from pCGN552 containing the ocs/KAN gene, pCGN587 was obtained.

Construction of pCGN739 (Binary Vector)

To obtain the gentamicin resistance marker, the gentamicin-resistance gene was isolated from a 3.1 kb EcoRI-PstI fragment of pPHIJ1 (Hirsch, et al., (1984), supra) and cloned into pUC9 (Vieira and Messing, Gene (1982) 19:259-268) yielding pCGN549. The HindIII-BamHI fragment from pCGN549 containing the gentamicin-resistance gene replaced the HindIII-BglII fragment of pCGN587, creating pCGN594. The pCGN594 HindIII-BamHI region, which contains a 5'-ocs-kanamycin-ocs-3' (ocs is octopine synthase with 5' designating the promoter region and 3' the terminator region, see co-pending U.S. application serial no. 834,161, filed February 26, 1986) fragment was replaced with the HindIII-BamHI polylinker region from pUC18 (Yanish-Peron, et al, Gene (1985) 33:103-119) to make pCGN739.

Construction of pCGN734

The HindIII-SphI fragment of pTiA6 corresponding to bases 3390-3241 (Barker, et al., Plant Mol. Biol. (1983) 2:335-350) was cloned into the HindIII-SphI site of M13mp19 (Yanisch-Perron, et al., Gene (1985) 53:103-119; Norrander, et al., Gene (1983) 26:101-106). Using an oligonucleotide corresponding to bases 3287 to 3300, DNA synthesis was primed from this template. Following S1 nuclease treatment and HindIII digestion, the resulting fragment was cloned in the HindIII-SmaI site of pUC19 (Yanisch-Perron, et al., Gene (1985) 33:103-119). (This resulted in an intermediate plasmid that should be named?) An EcoRI-HindIII fragment corresponding to bases 3287-3390 (Barker, et al., Plant mol. Biol. (1983) 2:335-350) was cloned with the EcoRI-

28

HindIII fragment of pTiA6 (corresponding to bases 3390-
4494) into the EcoRI site of pUC8 Vieira and Messing, Gene
(1982) 19:259-268) resulting in pCGN734.

5   Construction of pCGN726c

        pCGN556 contains the EcoRI-HindIII linker of pUC18
(Yanisch-Perron, et al., Gene (1985) 33:103-119) inserted
into the EcoRI-HindIII sites of pUC13-cm (K. Buckley
(1985), supra). The HindIII-BglII fragment of pNW31c-8,29-
10 1 (Thomashow, et al., Cell (1980) 19:729-739) containing
ORF1 and 2 of pTiA6 (Barker, et al., Plant Mol. Biol.
(1983) 2:335-350) was subcloned into the HindIII-BamHI
sites of pCGN556 producing pCGN703.

        The Sau3A fragment of pCGN703 containing the 3' region
15 of transcript 7 (corresponding to bases 2396-2920 of pTiA6
(Barker, et al., (1983) supra) was subcloned into the BamHI
site of pUC18 producing pCGN709. The EcoRI-SmaI polylinker
region of pCGN709 was replaced with the EcoRI-SmaI fragment
of pCGN587, which contains the kanamycin resistance gene
20 (APH3-II) producing pCGN726.

        The EcoRI-Sall fragment of pCGN726 plus the BglII-
EcoRI fragment of pCGN734 were inserted into the BamHI-SalI
site of pUC8-pUC13-cm (chloramphenicol resistant, K.
Buckely, (1985), supra) producing pCGN738. pCGN726c was
25 derived from pCGN738 by deleting the 900 bp EcoRI-EcoRI
fragment.

    Construction of pCGN528 (Shuttle Vector)

        pCGN525 was made by digesting a plasmid containing Tn5
30 which harbors a kanamycin gene (Jorgenson, et al., Mol.
Gen. Genet. (1979) 177:65) with HindIII-BamHI and inserting
the HindIII-BamHI fragment containing the kanamycin gene
into the HindIII-BamHI sites into the tetracycline gene of
pACYC184 (Chang and Cohen, J. Bacteriol. (1978) 134:1141-
35 1156). pCGN526 was made by inserting the BamHI fragment 19
of pTiA6 (Thomashow, et al., Cell (1980) 19:729-739),

modified with XhoI linkers inserted into the SmaI site, into the BamHI site of pCGN525. pCGN528 was obtained by deleting the small XhoI fragment from pCGN526 by digesting with XhoI and religating.

Construction of pCGN167

The AluI fragment of CaMV (bp 7144-7735) (Gardner, et al., Nucl. Acid Res. (1981) 9:2871-2888) was obtained by digestion with AluI. It was then cloned into the HincII site of M13mp7 (Messing, et al., Nucl. Acids Res. (1981) 9:309-321) to create C614. An EcoRI digest of C614 produced the EcoRI fragment from C614 containing the 35S promoter which was cloned into the EcoRI site of pUC8 (Vieira and Messing, Gene (1982) 19:259-268) to produce pCGN146. To trim the promoter region, the BglII site (bp 7670) was treated with BglII and resected with Bal31. Subsequently a BglII linker was attached to the Bal31 treated DNA to produce pCGN147.

pCGN148a, containing a promoter region, selectable marker (KAN with 2 ATG's) and 3' region, was prepared by digesting pCGN528 with BglII and inserting the BamHI-BglII promoter fragment from pCGN147. This fragment was cloned into the BglII site of pCGN528 so that the BglII site was proximal to the kanamycin gene of pCGN528.

pCGN149a was made by cloning the BamHI-kanamycin gene fragment from pMB9KanXXI into the BamHI site of pCGN148a. pMB9KanXXI is a pUC4 variant (Vieira and Messing, Gene (1982) 19:259-268) which has the XhoI site missing but contains a functional kanamycin gene from Tn903 to allow for efficient selection in Agrobacterium. pCGN149a was digested with BglII and SphI. This small BglII-SphI fragment of pCGN149a was replaced with the BamHI-SphI fragment from MI (see below) isolated by digestion with BamHI and SphI. This produces pCGN167, a construct containing a full length CaMV promoter., 1ATG-kanamycin gene, 3' end and the bacterial TN903-type kanamycin gene.

MI is an EcoRI fragment from pCGN550 (see construction of pCGN587) and was cloned into the EcoRI cloning site of M13mp9 so that the PstI site in the 1ATG-kanamycin gene was proximal to the polylinker region of M13mp9.

Construction of pCGN766c (35S promoter-3' region)

The HindIII-BamHI fragment of pCGN167 containing the CaMV-35S promoter, 1ATG-kanamycin gene and the BamHI-fragment 19 of pTiA6 was cloned into the BamHI-HindIII sites of pUC19 (Yanisch-Perron (1985), supra) creating pCGN976. The 35S promoter and 3' region from transcript 7 was developed by inserting a 0.7 kb HindIII-EcoRI fragment of pCGN976 (35S promoter) and the 0.5 kb EcoRI-SauI fragment of pCGN709 (transcript 7:3') into the HindIII-SalI sites of pCGN566 (pCGN566 contains the EcoRI-HindIII linker of pUC18 inserted into the EcoRI-HindIII sites of pUC13-Cm) creating pCGN766c.

Final Construction of pCGN783

The 0.7 kb HindIII-EcoRI fragment of pCGN766c (CaMV-35S promoter) was ligated to the 1.5 kb EcoRI-SalI fragment of pCGN726c (1ATG-Kan 3' region) followed by insertion into the HindIII-SalI fragment of pCGN778 containing the CaMV-35S promoter and 1ATG-KAN-3' region was used to replace the HindIII-SalI linker region of pCGN739 to produce pCGN783.

4.   Construction of pCGN2125, an EF-1α GUS Fusion Construct

To analyze the expression of 61-1 when re-introduced into tomato plants, the GUS (β-glucuronidase, E. coli) gene was inserted into the coding region of 61-1. The fusion was done so that the GUS coding region was in-frame with the EF-1α sequence so that a protein with GUS activity could be produced.

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a.   Construction of pCGN2125

To construct pCGN1804, the approximately 2 kb
BamHI-SstI fragment from pBI221 containing the GUS gene
(Jefferson, Plant Mol. Biol. Reporter (1987) 5:387-405) was
5   cloned into pUC119. To construct pCGN2124, pCGN2123 (see
Example II A.2.a.) was linearized with ClaI, and treated
with the Klenow fragment of DNA polymerase I to blunt the
ends. The resulting DNA was ligated to the BamHI-EcoRI
fragment of pCGN1804, containing the GUS gene, which had
10   also been treated with the Klenow fragment of DNA
polymerase. A clone, pCGN2124, was chosen that had this
fragment inserted in the correct orientation. The
ClaI/BamHI junction between EF-1α and GUS was sequenced to
verify that the GUS insertion was in-frame with the EF-1α
15   coding region. The fusion contained the first 34 amino
acids of EF-1α from 61-1 and 8 amino acids of linker region
followed by the GUS gene.

To facilitate transfer to tomato plants, pCGN2124 was
inserted into the Agrobacterium tumefaciens binary vector,
20   pCGN783 (see above). pCGN2124 was linearized with SalI and
inserted into the SalI site of pCGN783 (supra). A clone,
pCGN2125, with the EF-1α GUS gene being transcribed in the
same direction as the 35S-kan-tml gene was obtained. This
construct in the Agrobacterium tumefaciens LBA4404 (also
25   referred to as 2760) was used to transform tomato plants.

5.   Construction of pCGN7001 (mas-gus-mas construct)

To evaluate levels of GUS produced from pCGN2125
in tomato plants, tomato plants containing a mas-gus-mas
construct (pCGN7001) were generated. The mas (mannopine
30   synthase promoter) has been previously studied and shown to
give a relatively low level of expression (Murai and Kemp,
Nucl. Acids Res. (1982) 10:1679-1689).

To construct pCGN7001, a 5' mas-gus-mas 3' sequence
was inserted into the SalI site of pCGN783. The 5' mas-
35   gus-mas 3' construct contained sequentially the following
sequences: Xho linker; the NaeI to blunted ClaI fragment

of the T-DNA of pTiA6 which spans the nucleotides 20806 to
20128 (the numbering is by Barker, et al., _Plant Mol. Biol._
(1983) _2_:335-350 for the closely related Ti plasmid pTi
15955); the AccI to BamHI linker region of pUC19; the BamHI

5   to SstI fragment of pCGN1804 containing the β-glucuronidase
gene; the SstI to EcoRI linker region of pUC19; EcoRI
linker; the blunted HindIII to blunted SstI fragment of
pTiA6 which spans the nucleotides 19239 to 18462; (Barker,
et al., _supra_); Xho linker.

10   The 5' mas-gus-mas 3' sequence was inserted into
pCGN783 so that the 5' mas-gus-mas 3' sequence was
transcribed in the same direction as the 35S-kan-tml gene.
This construct in the _Agrobacterium tumefaciens_ LBA4404 was
used to transform tomato plants.

15

        6.   Transformation of Tomato Cotyledon Tissue
        a.   Preparation of A. tumefaciens LBA
4404/pCGN7001 and pCGN2125

            The plasmids pCGN7001 or pCGN2125, respectively,

20   were transformed into _E. coli_ C2110 (polA) as follows.
        Bacterial matings were performed using two _E. coli_
strains and _Agrobacterium tumefaciens_ strain LBA4404. One
E. coli strain (MM294) harbored pRK2073 which provided
mobilization functions and the other strain (C2110) carried

25   the plasmid with a kanamycin resistance marker to be
transferred into _Agrobacterium_. The two _E. coli_ strains
were grown overnight at 37°C with shaking in LB broth. The
Agrobacterium strain was grown overnight at 28°C in MG/L
broth. Fifty microliters of each of the three strains were

30   mixed on a nitrocellulose filter placed on an MG/L plate.
The plate was incubated at 28C for 3 days. The mixture was
then streaked onto an AB minimal medium supplemented with
100μg/ml kanamycin and 100μg/ml streptomycin and incubated
at 28°C for two days. The streptomycin was included to

35   kill the two _E. coli_ strains. Single colonies were picked
and purified by two more streakings on the above medium.

b.   Transformation of Tomato Cotyledon Tissue with
pCGN7001 and pCGN2125

Substantially sterile tomato cotyledon tissue was
obtained from seedlings which had been grown at 24°C, with
5   a 16 hr/8 hr day/night cycle in 100 x 25mm Petri dishes
containing Murashige-Skoog salt medium and 0.8% agar (pH
6.0). Any tomato species may be used, however, here the
inbred breeding line was UC82B, available from the
Department of Vegetable Crops, University of California,
10   Davis, CA 95616. The cotyledons were cut into three
sections and the middle section placed onto feeder plates
for a 24-hour preincubation. The feeder plates were
prepared by pipetting 0.5ml of a tobacco suspension culture
(~10^6 cells/ml) onto 0.8% agar medium, containing Murashige
15   minimal organic medium (K.C. Biologials), 2,4-D (0.2mg/L),
kinetin (0.1mg/L), thiamine (0.9mg/L) and potassium acid
phosphate (200mg/L, pH 5.5). The feeder plates were
prepared two days prior to use. A sterile 3mm filter paper
disk containing feeder medium was placed on top of the
20   tobacco cells after the suspension cells had grown for two
days.

Following the preincubation period, the middle
segments of the cotyledon sections were placed into a
liquid MG/L broth culture (1-5ml) of the _Agrobacterium_
25   _tumefaciens_ strain LBA4404/pCGN7001 or pCGN2125 (1x10^7-
1x10^8 bacteria/ml). The cotyledon sections were
cocultivated with the bacteria for 48 hrs. on the feeder
plates and then transferred to regeneration medium
containing 350mg/L cefotaxime and 100mg/L kanamycin. The
30   regeneration medium is a K.C. Biologicals Murashige-Skoog
salts medium with zeatin (2mg/L), myo-insitol (100mg/L),
sucrose (20g/L), Nitsch vitamins and containing 0.8% agar
(pH 6.0). In 2 to 3 weeks, shoots developed. When the
shoots were approximately 1.25cm, they were excised and
35   transferred to a Murashige and Skoog medium containing

cefotaxime (350mg/L) and kanamycin (50mg/L for rooting.
Roots developed within 10-12 days.

 7. Analysis of GUS Expression in Transformed Tomato
5 Seedlings
 Young leaf tissue was excised from tomato
plantlets that were growing from the cut edges of
cotyledons that had been co-cultivated with Agrobacterium
tumefaciens containing pCGN2125 or pCGN7001 constructs 6-8
10 weeks previously. The tissue was grown on medium
containing 100mg/ml kanamycin; 350mg/ml cefotaxime (Cal
Biochem-Hoechst). The remainder of the medium was 22
medium (Murashige and Skoog salts 2% sucrose, 100mg/l myo-
inositol, Nitsch vitamins, 2mg/l zeatin and 0.8%
15 Bactoagar). One plantlet from cocultivation with pCGN7001
were chosen for further analysis.
 The fluorimetric GUS assay was performed essentially
as described (Jefferson et al., EMBO J. (1987) 6:3901-
3907). Specifically, young tomato leaf tissue (0.03-0.20
20 g) was ground in 500µl GUS extraction buffer (50mM sodium
phosphate, pH 7.0, 10mM BME, 10mM EDTA, 0.1% Triton X-100).
The extract was centrifuged in a microfuge for 10 min. and
the supernatant removed. Various dilutions of the
supernatant in extraction buffer were made. Typically 1:10
25 dilutions in 500µl total volume were analyzed. Five µl of
MUG (methylumbelliferone), 100mM in dimethylformamide, were
added and the reaction mixtures incubated at 37°C for 2
hours. The reaction was terminated by the addition of
900µl of 0.2 M Na_2CO_3. Fluorescence was measured with
30 excitation at 365 nm, emission at 455 nm on a Farrand
system 3 fluorimeter with slit widths set at 10 nm.

 B. Results
 1. Characterization of a EF-1α Genomic Clone
35 The EF-1α gene contains one intron of 320 bp. This
gene encodes a polypeptide which is 97% homologous to the

35

polypeptide encoded by LeEF-1. 61-1 is therefore one of the members of the EF-1α multigene family, but it is not the gene that gave rise to the LeEF-1 cDNA clone. The sequence of the EF-1α genomic clone, 61-1, is shown in

5   Figure 3.

    To determine what percentage of the total EF-1 mRNA arises from the 61-1 gene and what percentage arises from the gene that gave rise to LeEF-1 (pCGN666), specific oligomers were synthesized. Oligomer EF p27 (5'-

10 AGCTTGTAGATCAAGTCACCAGTGGTAGTC-3') corresponds to bases 123 to 153 of LeEF-1 and since it is in a conserved region of the coding sequence will bind to all EF-1α mRNAs. Oligomer EF p28 (5'-ACTGATATCTCTGGTAACTCCAGAGTTGAA-3') corresponds to bases 2542 to 2572 of 61-1 in the 3'-untranslated

15 region. This sequence is unique to 61-1. Oligomer EF p29 (5'-ATAGCATTCGAAACACCAGCATCACACTGC-3') corresponds to bases 1413 to 1443 of LEF-1 i the 3'-untranslated region. This sequence is unique to LeEF-1.

    Using these three oligomer probes, Northern analysis

20 was run on poly(A+) RNA from young axial shoots of tomato. The results demonstrated that the gene that gave rise to LeEF-1 (pCGN666) contributes a major portion of the EF-1α message. Comparing signals obtained with oligomers EFp27 and EFp29, it is possible to estimate that the gene that

25 gave rise to LeEF-1 contributes at least 30% and perhaps as much as 50-60% of the total EF-1α mRNA. On the other hand, a comparison of the signals obtained from EFp27 and EFp28, indicates that 61-1 contributes on the order of only 1% of the total EF-1α mRNA.

30   2.   Analysis of Tomato Plants Transformed with pCGN2125

    The results of a typical assay are shown in Table 1 below.

<table>
<tr><td colspan="4" style="text-align:center"><u>Table 1.</u>
<u>FLUOROMETRIC GUS ASSAY</u></td></tr>
</table>

Plant Sample	Weight of Sample	Fluorescence Intensity (F) of 1/10 Dilution	Fluorescence Intensity (F) normalized to 0.1g Sample
2125-X	0.04g	163	408
7001-A	0.14g	311	222

The GUS levels produced by the 2125 (EF-1α GUS) plant
are similar to those produced by a 7001 (mas-gus) plant.
The level of EF-1α mRNA produced by the native 61-1 gene is
approximately 1% of the total EF-1α RNA. Since the total
EF-1α mRNA level is approximately 1% of the poly(A$^+$) RNA,
then the mRNA produced by the 61-1 gene represents about
0.01% of the poly(A$^+$) RNA. The message produced from the
mannopine synthase promoter is low level and may be equal
to or less than that produced from the octopine synthase
(ocs) promoter (Murai and Kemp, <u>Nucl. Acids Res.</u> (1982)
<u>10</u>:1679-1689). The level of mRNA produced from the ocs
promoter averages around 0.005% and ranges from 0.002% to
0.01% of total mRNA (Comai, et al., <u>Nature</u> (1985) <u>317</u>:741-
744). Thus the mannopine promoter and the 61-1 EF-1α gene
produce similar levels of mRNA. Thus the similar levels of
GUS activity produced from the 2125 and 7001 plants are as
would be predicted and indicate that the EF-1α-GUS fusion
in 2125 is functioning as expected.

Example III
EF-1α Genomic Clone A, The Genomic
Equivalent of pCGN666

5   A.   Materials and Methods

1.   Construction of pCGN2127

The approximately 250 bp XbaI (1464) to EcoRI fragment
of pCGN666 was cloned into XbaI-EcoRI digested pUC19. This
resulted in pCGN2127.

10   2.   Probe Description, Isolation and Labelling

Probe A: The 1.7 kb EcoRI fragment of pCGN666
containing LeEF-1 was isolated and nick-translated as
previously described (Shewmaker, et al., supra).

Probe B: The approximately 250 bp XbaI-EcoRI
15 fragment of pCGN2127 was isolated and nick-translated as
previously described (Shewmaker, et al., supra). This
fragment contains only sequences in the 3'untranslated
portion of LeEF-1.

Probe C: The 30 base oligonucleotide EFp29 (see
20 supra) which hybridizes to nucleotides 1413 to 1443 of
LeEF-1. This oligonucleotide hybridizes to sequences in
the 3' untranslated region of LeEF-1 that are different
from those to which probe B hybridizes.

25   3.   Screening a Tomato Genomic Library for the
Genomic Equivalent to pCGN666

A tomato (Lycopersicon escultentum cv VFNT-Cherry)
partial Sau3A library in Ch35 (Loenen, Gene (1983) 26:171-
179) was provided by Dr. Robert Fischer of University of
30 California, Berkeley. The basic screening procedures were
as described above in Example II. When the nick-translated
probes A and B were used, hybridization and washing were as
described for genomic screening. When the oligonucleotide
probe C was used, hybridization and washing were as
35 described for Northern analysis using oligonucleotide
probes.

Approximately 800,000 plaques of the above library
were screened with probe A. Subsequent rounds of plaque
purification of positive signals were done with duplicate
filters, one hybridized with probe A and the other with

5   probe B. Only plaques that hybridized to both probes were
chosen for final plaque purification. Purified plaques
that hybridized to both probes A and B were also screened
with probe C. A clone denoted "A" that hybridized to all 3
probes was chosen for further analysis. The EF-1α gene

10 within A was designated LeEF-A.

          4. Mapping, Subcloning and Sequencing
     DNA from phage "A" was isolated as described
(Grossgerger, Nuc. Acids. Res. (1987) 15:6737). The 11 kb

15 insert of tomato genomic DNA within "A" and the location of
LeEF-A within this insert were mapped using standard
techniques. (Maniatis, Fritsch and Sambrock, Molecular
Cloning, A Laboratory Manual (1982) Cold Spring Harbor, New
York) Several subclones of the insert in "A" were

20 generated using standard cloning techniques. These
subclones were used for DNA sequencing and included.
     pCGN674 and pCGN675: The approximately 9 kb KpnI
fragment cloned in both orientations in pUC19. This
fragment contains 5' upstream regions as well as coding

25 region from the amino terminal portion of the gene.
     pCGN2134: The approximately 2.5 b EcoRI fragment
cloned into pUC19. This fragment contains the coding
region and region 3' of the LeEF-A gene.
     Using pCGN674, pCGN674, pCGN2134 and standard

30 sequencing techniques similar to those described supra, the
sequence of (1) the coding region, (2) approximately 2.5 kb
upstream of the initiation codon, and (3) approximately 0.7
kb 3' of the stop codon were obtained.

5.   Construction of pCGN1558

pCGN1558 is a binary plant transformation vector
containing the left and right T-DNA borders of
Agrobacterium tumefaciens octopine Ti-plasmid pTiA6
5   (Currier and Nester *J. Bact.* (1976) 126:157-165), the
gentamycin resistance gene of pPH1JI (Hirsch and Beringer,
Plasmid (1984) 12:139-141), an *Agrobacterium rhizogenes* Ri
plasmid origin of replication from pLJbB11 (Jouanin et al.,
Mol. Gen. Genet. (1985) 201:370-374), a 35S promoter-Kan^R-
10   tml3 region capable of conferring kanamycin resistance to
transformed plants, a ColE1 origin of replication from
pBR322 (Bolivar et al., *Gene* (1977) 2:95-113), and a *lacZ'*
screenable marker gene from pUC18 (Yanish-Perron, et al.,
Gene (1985) 33:103-119).

15   Construction of pCGN1546

The 35S promoter-tml3' expression cassette, pCGN986
(contains a cauliflower mosaic virus 35S (CaMV35) promoter
and a T-DNA tml 3'-region with multiple restriction sites
between them. pCGN986 was derived from another cassette,
20   pCGN206, containing a CaMV35S promoter and a different 3'
region, the CaMV region VI 3'-end. The CaMV 35S promoter
was cloned as an *AluI* fragment (bp 7144-7734) (Gardner et.
al., *Nucl. Acids Res.* (1981) 9:2871-2888) into the *HincII*
site of M13mp7 (Messing, et. al., *Nucl. Acids Res.* (1981)
25   9:309-321) to create C614. An *EcoRI* digest of C614
produced the *EcoRI* fragment from C614 containing the 35S
promoter which was cloned into the *EcoRI* site of pUC8
(Viera and Messing, *Gene* (1982) 19:259) to produce pCGN147.

pCGN148a containing a promoter region, selectable
30   marker (KAN with 2 ATG's) and 3' region, was prepared by
digesting pCGN528 with *BglII* and inserting the *BamHI-BglII*
promoter fragment from pCGN147. This fragment was cloned
into the *BglII* site of pCGN528 so that the *BglII* site was
proximal to the kanamycin gene of pCGN528.

35   The shuttle vector used for this construct pCGN528,
was made as follows: pCGN525 was made by digesting a

40

plasmid containing Tn5 which harbors a kanamycin gene
(Jorgenson et. al., Mol. Gen. Genet. (1979) 177:65) with
HindIII-BamHI and inserting the HindIII-BamHI fragment
containing the kanamycin gene into the HindIII-BamHI sites
5   in the tetracycline gene of pACYC184 (Chang and Cohen, J.
Bacteriol. (1978) 134:1141-1156). pCGN526 was made by
inserting the BamHI fragment 19 of pTiA6 (Thomashow et.
al., Cell (1980) 19:729-739), modified with XhoI linkers
inserted into the SmaI site, into the BamHI site of
10 pCGN525. pCGN528 was obtained by deleting the small XhoI
fragment from pCGN526 by digesting with XhoI and
religating.

pCGN149a was made by cloning the BamHI-kanamycin gene
fragment from pMB9KanXXI into the BamHI site of pCGN148a.
15 pMB9KanXXI is a pUC4K variant (Vieira and Messing, Gene
(1982) 19:259-268) which has the XhoI site missing, but
contains a functional kanamycin gene from Tn903 to allow
for efficient selection in Agrobacterium.

pCGN149a was digested with HindIII and BamHI and
20 ligated to pUC8 digested with HindIII and BamHI to produce
pCGN169. This removed the Tn903 kanamycin marker. pCGN565
and pCGN169 were both digested with HindIII and PstI and
ligated to form pCGN203, a plasmid containing the CaMV 35S
promoter and part of the 5'-end of the TN5 kanamycin gene
25 (up to the PstI site, Jorgenson et. al., (1979), supra). A
3'-regulatory region was added to pCGN203 from pCGN204 (an
EcoRI fragment of CaMV (bp 408-6105) containing the region
VI 3' cloned into pUC18 (Yanisch-Perron, et al., Gene
(1985) 33:103-119) by digestion with HindIII and PstI and
30 ligation. The resulting cassette, pCGN206, was the basis
for the construction of pCGN986.

The pTiA6 T-DNA tml 3'-sequences were subcloned from
the Bam19 T-DNA fragment (Thomashow et al., (1980) supra)
as a BamHI-EcoRI fragment (nucleotides 9062 to 12,823,
35 numbering as in Barker et al., Plant Mol. Biol. (1982)
2:335-350) and combined with the pACYC184 (Chang and Cohen

(1978), _supra_) origin of replication as an EcoRI-HindIII fragment and a gentamycin resistance marker (from plasmid pLB41), obtained from D. Figurski) as a BamHI-HindIII fragment to produce pCGN417.

5   The unique SmaI site of pCGN417 (nucleotide 11,207 of the Bam19 fragment) was changed to a SacI site using linkers and the BamHI-SacI fragment was subcloned into pCGN565 to give pCGN971. The BamHI site of pCGN971 was changed to an EcoRI site using linkers. The resulting

10   EcoRI-SacI fragment containing the tml 3' regulatory sequences was joined to pCGN206 by digestion with EcoRI and SacI to give pCGN975. The small part of the Tn5 kanamycin resistance gene was deleted from the 3'-end of the CaMV 35S promoter by digestion with SalI and BglII, blunting the

15   ends and ligation with SalI linkers. The final expression cassette pCGN986 contains the CaMV 35S promoter followed by two SalI sites, an XbaI site, BamHI, SmaI, KpnI and the tml 3' region (nucleotides 11207-9023 of the T-DNA).

    The 35S promoter-tml 3' expression cassette, pCGN986

20   was digested with HindIII. The ends were filled-in with Kleenow polymerase and XhoI linkers added. The resulting plasmid was called pCGN986X. The BamHI-SacI fragment of pBRX25 containing the nitrilase gene was inserted into BamHI-SacI digested pCGN986X yielding pBRX66.

25   Construction of pBRX25 is described in European application EPA 0 229 042 filed January 7, 1987, which application is incorporated herein by reference. Briefly, the method was as follows: The nucleotide sequence of a 1212-bp PstI-HincII DNA segment encoding the bromoxynil-

30   specific nitrilase contains 65-bp of 5' untranslated nucleotides. To facilitate removal of a portion of these excess nucleotides, plasmid pBRX9 was digested with PstI, and treated with nuclease Bal31. BamHI linkers were added to the resulting ends. BamHI-HincII fragments containing a

35   functional bxn gene were cloned into the BamHI-SmaI sites

42

of pCGN565. The resulting plasmid, pBRX25, contains only 11 bp of 5' untranslated bacterial sequence.

pBRX66 was digested with PstI and EcoRI, blunt ends generated by treatment with Kleenow polymerase, and XhoI

5 linkers added. The resulting plasmid pBRX68 now has a tml 3' region that is approximately 1.1kb. pBRX68 was digested with SalI and SacI, blunt ends generated by treatment with Kleenow polymerase and EcoRI linkers added. The resulting plasmid, pCGN986XE is a 35S promoter - tml 3' expression

10 cassette lacking the nitrilase gene.

The Tn5 kanamycin resistance gene was then inserted into pCGN986XE. The 1.0kb EcoRI fragment of pCGN1536 (see below) was ligated into pCGN986XE digested with EcoRI. A clone with the Tn5 kanamycin resistance gene in the correct

15 orientation for transcription and translation was chosen and called pCGN1537b. The 35S promoter Kan^R-tml 3' region was then transferred to a chloramphenical resistant plasmid backbone. pCGN786 (a pUC-CAM based vector with the synthetic oligonucleotide 5'

20 GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3' containing the cloning sites EcoRI, SalI, BglII, PstI, XhoI, BamHI, and HindIII inserted into pCGN566) was digested with XhoI and the XhoI fragment of pCGN1537b containing the 35S promoter - Kan^R-tml 3' region was ligated in. The

25 resulting clone was termed pCGN1546.

pCGN1536 Construction.

The 5.4 kb EcoRI fragment is removed from pVK232 (Knauf and Nester, Plasmid (1982) 8:45), by EcoRI digestion and cloned into EcoRI digested pACYC184 (Chang and Cohen,

30 J. Bacteriol. (1978) 134:1141-1156) to create pCGN14. The 1434 bp ClaI-SphI fragment of pCGN14, containing the mas 5' region (bp20128-21562 according to numbering of (Barker et al., Plant Mo. Biol. (1983) 2:335-350) is cloned into AccI-SphI digested pUC19 (Yanisch-Perron et al., Gene (1985)

35 33:103-119) to generate pCGN40. A 746 bp EcoRV-NaeI fragment of the mas 5' region is replaced by an XhoI site

43

by digesting pCGN40 with EcoRV and NaeI followed by
ligation in the presence of a synthetic XhoI linker DNA to
create pCGN1036. The 765 bp SstI-HindIII fragment (bp
18474-19239) of pCGN14, containing the mas 3' region, is
5 cloned into SstI-HindIII digested pUC18 (Yanish-Perron et
al., Gene (1985) 33:103-119) to yield pCGN43. The HindIII
site of pCGN43 is replaced with an EcoRI site by digestion
with HindIII, blunt ending with Klenow enzyme, and ligation
of synthetic EcoRI linker DNA to create pCGN1034. The 767
10 bp EcoRI fragment of pCGN1034 is cloned into EcoRI-digested
pCGN1036 in the orientation that places bp 19239 of the mas
3' region proximal to the mas 5' region to create pCGN1040.
pCGN1040 is subjected to partial digestion with SstI,
treated with T4 DNA polymerase to create blunt ends, and
15 ligated in the presence of synthetic XhoI linker DNA; a
clone is selected in which only the SstI site at the
junction of bp 18474 and vector DNA (constructed in pCGN43
and carried into pCGN1040) is replaced by an XhoI site to
generate pCGN1047.

20   pCGN565 is digested with EcoRI and HindIII, treated
with Klenow enzyme to create blunt ends, and ligated in the
presence of synthetic XhoI linker DNA to create pCGN1003;
this recreates the EcoRI site adjacent to the XhoI linker.
pCGN1003 is digested with EcoRI, treated with Klenow enzyme
25 to create blunt ends, and ligated in the presence of
synthetic PstI linker DNA to create pCGN1007. The 1.5kb
XhoI fragment of pCGN1047, containing the mas 5' region and
the mas 3' region with a multiple cloning site between, is
cloned into XhoI digested pCGN1007 to construct pCGN1052.
30 A portion of the multiple cloning site of pCGN1052 is
deleted by digestion with XbaI and SstI, treated with
Klenow enzyme to make blunt ends, and ligated to generate
pCGN1052ΔXS.

The 1 kb EcoRI-SmaI fragment of pCGN550 (See pCGN783,
35 Example II), containing the 1 ATG-kanamycin resistance
gene, is cloned into EcoRI-SmaI digested Bluescript M13-KS

(Strategene, Inc., CA) to create pBSKm; this plasmid
contained an M13 region allowing generation of single
stranded DNA. Single stranded DNA is generated according
to the supplier's recommendations, and in vitro mutagenesis
5 was performed (Adelman et al., DNA (1983) 2:183-193) using
a synthetic oligonucleotide with the sequence
5'GAACTCCAGGACGAGGC3' to alter a PstI site with the
kanamycin resistance gene and make it undigestable,
creating pCGN1534. pCGN1534 is digested with SmaI and
10 ligated in the presence of synthetic EcoRI linker DNA to
generate pCGN1535.

The 1 kb EcoRI fragment of pCGN1535 is cloned into
EcoRI digested pCGN1052ΔXS to create the mas5'-kan mas3'
plant selectable marker cassette pCGN1536.

15 Construction of pCGN1541b

pCGN65α2XX (see below) was digested with BglII and
EcoRV, treated with Kleenow polymerase, and BglII linkers
added. The resulting plasmid, pCGN65α2XX', now lacks an
approximately 20 bp piece of DNA that was present in
20 pCGNα2XX.

pBR322 (Bolivar et al., Gene (1977) 2:95-113) was
digested with EcoRI and PvuII, treated with Kleenow
polymerase to generate blunt ends, and BglII linkers added.
An ampicillin resistant, tetracycline sensitive clone,
25 pCGN1538 was selected. This clone now lacks the
approximately 2.2 kb EcoRI-PvuII fragment containing the
tetracycline resistance gene. The PvuII site has been lost
but the EcoRI site was regenerated upon addition of BglII
linkers.

30 pCGN65α2XX' was digested with BglII and ligated to
BglII digested pCGN1538 to create pCGN1541a which contained
both plasmid backbones. pCGN1541a was digested with XhoI
and religated. An ampicillin resistant, chloramphenical
sensitive clone was chosen which lacked the pACYC-184
35 derived backbone, creating pCGN1541b.

45

Construction of pCGN65α2XX

pCGN451 consists of octopine synthase 5' non-coding region sequences 13,643 to 15,208 fused via an EcoRI linker to the 3' non-coding region sequences 11,207 to 12,823 of

5   pTiA6 according to Barker et al., Plant Mol. Biol. (1983) 2:325. pCGN451 was digested with HpaI and ligated in the presence of synthetic SphI linker DNA to generate pCGN55. The XhoI-SphI fragment of pCGN55 (bp 13800-15208, including the right border, of Agrobacterium tumefaciens T-DNA;

10   (Barker et al., Gene (1977) 2:95-113) was cloned into SalI-SphI digested of pUC19 (Yanisch-Perron et al., Gene (1985) 33:103-119) to create pCGN60. The 1.4 kb HindIII-BamHI fragment of pCGN60 was cloned into HindIII-BamHI digested with pSP64 (Promega; Inc.) to generate pCGN1039. pCGN1039

15   was digested with SmaI and NruI (deleting bp14273-15208; (Barker et al., Gene (1977) 2:95-113) and ligated in the presence of synthetic BglII linker DNA creating pCGN1039ΔNS. The 0.47 kb EcoRI-HindIII fragment of pCGN1039ΔNS was cloned in to EcoRI-HindIII digested

20   pCGN565 to create pCGN565RB. The HindIII site of pCGN565RB was replaced with an XhoI site by HindIII digestion, treatment with Klenow enzyme, and ligation in the presence of synthetic XhoI linker DNA to create pCGN565RB-H+X.

      pUC18 was digested with HaeII to release the lacZ'

25   fragment, treated with Klenow enzyme to create blunt ends, and the lacZ'-containing fragment ligated into pCGN565RB-H+X, which had been digested with AccI and SphI and treated with Klenow enzyme, resulting in pCGN565RB α2X. In pCGN565RBα2X the orientation of lacZ' is such that the lac

30   promoter is proximal to the right borderThe clone is positive for lacZ' expression when plated on an appropriate host and contains bp 13990-14273 of the right border fragment (Barker et al. (1983) supra), having deleted the AccI-SphI fragment (bp 13800-13990). The 728 bp BglII-XhoI

35   fragment of pCGN565RBα2X containing the T-DNA right border piece and the lacZ' gene, was cloned into BglII-XhoI

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digested pCGN65ΔKX-S+X, replacing the BglII-XhoI right
border fragment of pCGN65ΔKX-S+X, to create pCGN65α2X.

   Construction of pCGN65ΔKX-S+X

   pCGN501 was constructed by cloning a 1.85 kb EcoRI-
5   XhoI fragment of pTiA6 (Currier and Nester, J. Bact. (1976)
126:157-165) containing bases 13362-15208 (Barker et al.,
Plant Mo. Biol. (1983) 2:335-350) of the T-DNA (right
border), into EcoRI-SalI digested M13mp9 (Vieira and
Messing, Gene (1982) 19:259-268). pCGN502 was constructed
10 by cloning a 1.6 kb HindIII-SmaI fragment of pTiA6,
containing bases 602-2212 of the T-DNA (left Border), into
HindIII-SmaI digested M13mp9. pCGN501 and pCGN502 were
both digested with EcoRI and HindIII and both T-DNA-
containing fragments cloned together into HindIII digested
15 pUC9 (Vieira and Messing, Gene (1982) 19:259-268) to yield
pCGN503, containing both T-DNA border fragments. pCGN503
was digested with HindIII and EcoRI and the two resulting
HindIII-EcoRI fragments (containing the T-DNA borders) were
cloned into EcoRI digested pHC79 (Hohn and Collins, Gene
20 (1980) 11:291-298) to generate pCGN518. The KpnI-EcoRI
fragment from pCGN518, containing the left T-DNA border, is
cloned into KpnI-EcoRI digested pCGN565 to generate
pCGN580. The BamHI-BglII fragment of pCGN580 is cloned
into the BamHI site of pACYC184 (Change and Cohen, J.
25 Bacteriol. (1978) 134:1141-1156) to create pCGN51. The 1.4
kb BamHI-SphI fragment of pCGN60 (see pCGN65α2X section
above) containing the T-DNa right border fragment, was
cloned into BamHI-SphI digested pCGN51 to create pCGN65.

   pCGN65 was digested with KpnI and XbaI, treated with
30 Klenow enzyme to create blunt ends, and ligated in the
presence of synthetic BglII linker DNA to create pCGN65ΔKX.
pCGN65ΔKX was digested with SalI, treated with Klenow
enzyme to create blunt ends, and ligated in the presence of
synthetic XhoI linker DNA to create pCGN65ΔKX-S+X.

35

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Final Construction of pCGN1558

The XhoI fragment of pCGN1546 containing the 35S promoter - KanR-tml 3' region was cloned into XhoI digested 1541b to create pCGN1554. The XhoI fragment from pCGN1546 is oriented within 1541b such that the order of components is <left border - tml 3' - KanR - 35S promoter - lacZ'> <right border.

The BglII fragment of pCGN1554 containing left border-tml3'-KanR-35S promoter-lacZ right border was cloned into BamHI restricted pCGN1532 (see below) resulting in the binary vector, pCGN1558. In pCGN1558, the orientation of the insert is such that the T-DNA left border is adjacent to the RI plasmid origin of replication. This binary vector has several advantages including a minimal amount of DNA between the T-DNA borders, high stability in Agrobacterium hosts, high copy number in E. coli hosts and blue/white screen with multiple restriction enzyme sites for ease of cloning target DNA.

pCGN1532 construction.

The 3.5kb EcoRI-PstI fragment containing the gentamycin resistance gene is removed from pPh1JI (Hirsch and Beringer, Plasmid (1984) 12:139-141) by EcoRI-PstI digestion and cloned into EcoRI-PstI digested pUC9 (Vieira and Messing, Gene (1982) 19:259-268) to generate pCGN549. HindIII-PstI digestion of pCGN549 yields a 3.1 kb fragment bearing the gentamycin resistance gene, which is made blunt ended by the Klenow fragment of DNA polymerase I and cloned into PvuII digested pBR322 (Bolivar et al., Gene (1977) 2:95-113) to create pBR322Gm. pBR322Gm was digested is DraI and SphI, treated with Klenow enzyme to create blunt ends, and the 2.8 kb fragment cloned into the Ri origin containing plasmid pLJbB11 (Jouanin et al., Mol. Gen. Genet. (1985) 201:370-374) which has been digested with ApaI and made blunt ended with Klenow enzyme, creating pLHbB11Gm. The extra ColE1 origin and the kanamycin resistance gene are deleted from pLJvB11GM by digestion

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with BamHI followed by self closure to create pGmB11. The
HindII site of pGmB11 is deleted by HindII digestion
followed by treatment with Klenow enzyme and self closure,
creating pGmB11-H. The PstI site of pGmB11-H is deleted by
5 PstI digestion followed by treatment with Klenow enzyme and
self closure, creating pCGN1532.

6. <u>Construction of EF-1α (Le-EFA)-GUS Construct and Plant
Transformation</u>

10   a.   <u>Construction of pCGN2147 – EF-1α 3' subclone for
use in EF-1α cassette</u>

pCGN2141 was constructed by cloning the approximately
1.8kb KpnI-PstI fragment of pCGN2134 into pUC19 cut with
KpnI x PstI. This KpnI (Figure 4, nucleotide #2771) – PstI
15 fragment contains coding and 3' untranscribed regions. The
approximately 1.0kb BglII-PstI fragment of 2141 was cloned
into Bluescript KSII (Stratagene) that has been digested
with BamHI x PstI yielding pCGN2145. This BglII (Figure 4,
nucleotide #3619) – Pst fragment contains C-terminal coding
20 regions and 3' untranscribed regions. The 1.0kb SstI-EcoRI
fragment of 2145 was cloned into SstI-EcoRI digested
pCGN565 yielding pCGN2147.

    b.   <u>Construction of 2140-EF-1α 5' Promoter Subclone
For Use In EF-1α Cassette</u>

25   In order to obtain desirable restriction sites for
cloning in genes at the 3' end of a promoter fragment, a
small fragment of the 5' region of EF-1α was amplified
using polymerase chain reaction (PCR) (Perkin-Elmer, Cetus)
thermal cycler, as per the manufacturer's instructions
30 (including manufacturer's reagents) and oligonucleotides
that add desired linkers. This small fragment was then
rejoined with other 5' sequences to make a suitable
promoter fragment. Specifically the oligos were:

EFp54

HindIII

|--------|

5'   CAG - CAG - AAG - CTT - GCT - GTA - GTT - GTT-

5   2135

TAC - CAG 3'   .

2158

10   EFp56

Sst   Sma

|-------| |-------|

5'   ACG - ACG - GAG - CTC - TCG - CCC - GGG

15

Xho

|-------|

- CAT - CTC - GAG - ACA - CTA - AGA - AAC

20   2450

TGC - ATT - TAC - 3'

2427

25

        Specifically the reaction contained 53.5ul H2O, 10ul
10xRxn buffer, 16ul dNTP's [1.25mM dCTP, dATP, dGTP & TTP],
5ul EF-p54 (20mM), 5ul EF-p56 (20mM), 20ul pCGN675
(1ng/ul), 0.5ul TAQ polymerase. The resulting ≈ 300bp

30 fragment was restricted with HindIII and SstI and cloned
into pUC12 CmR (Buckley, Ph.D. thesis) that had been cut
with HindIII and SstI. This resulted in pCGN2142. The
sequence of the fragment in 2142 generated by PCR was
determined and found to be identical to the same region in

35 pCGN675 (See Example 3) (Figure 4, nucleotide #2135-->
2450).

pCGN2137 was obtained by cutting pUC19 with HindIII, filling in with Kleenow polymerase and selecting for a clone that had lost the HindIII site.

The approximately 6.5kb BamHI-KpnI (2771) fragment of
5   pCGN675 was cloned into BamHI x KpnI cut pCGN2137 yielding pCGN2139. The BamHI-KpnI fragment in pCGN2139 contains 5' upstream regions and approximately 300 base pairs of N-terminal coding region. The approximately 9.5kb HindIII-SstI fragment of 2139 was ligated to the approximately
10 300bp HindIII-SstI fragment of pCGN2142 yielding pCGN2140. The approximately 6kb EF-1α 5' fragment in 2140 contains 5' upstream and 5' untranslated regions but no coding sequences.

c.   Construction of pCGN2148-EF-1α Cassette
15   The approximately 6kb SphI-SstI fragment of 2140 containing the EF-1α promoter was cloned into SphI-Sst cut pCGN2147 yielding pCGN2148. This clone thus contains the approximately 6kb 5' promoter fragment, the approximately 1kb 3' EF-1α fragment and restriction sites in between into
20 which genes can be cloned.

d.   Construction of pCGN2154-EF-1α-GUS Chimera
pCGN1804 (see Example II) contains the E. coli β-glucuronidase (GUS) gene. pCGN1804 was cut with EcoRI and the ends filled in with polymerase I (Focus (1984) 6:1 pg.
25 6). XhoI linkers were added and a clone with an XhoI site was selected and designated pCGN1805. The 1.8kb SalI x XhoI fragment of pCGN1805 containing the GUS gene was cloned into XhoI digested pCGN2148. A clone with the GUS gene in the correct orientation for transcription and
30 translation was selected and given the number pCGN2154.

e.   Insertion of EF-1α GUS Chimera (pCGN2154) Into A Binary Vector (pCGN1558)
The approximately 9kb PstI fragment of pCGN2154 containing EF-1α 5'-GUS-EF-1α3' was cloned into PstI cut
35 pCGN1558 (see above). In pCGN2158 the 5'EF-1α-GUS EF-1α3' was inserted into pCGN1558 such that 5'EF-1α-GUS-EF-1α3' is

transcribing in the same direction as the 35S-Kan-tml gene. In pCGN2157 the 5'EF-1α-GUS-EF-1α3' was inserted into pCGN1558 such that 5'EF-1α-GUS-EF-1α3' is transcribing in the opposite direction as the 35S-Kan-tml gene.

5   f.   Preparation of Transgenic Plants

Agrobacterium tumefaciens strain 2760 (also known as LBA4404, Hoekerana et al., Nature (1983) 303:179-180) was transformed with pCGN2157 or pCGN2158 using the method of Holsters, et al., Mol. Gen. Genet., (1978) 163:181-187.

10   The transformed A. tumefaciens was then used in the co-cultivation of plants.

Tomato

Tomato plants were transformed with A. tumefaciens LBA4404/pCGN2158 or pCGN2157, respectively, essentially as

15   described in Example II. 6.b., with the exception that 500 mg/L of carbenicillin was used in the co-cultivation and regeneration procedure instead of the 350 mg/l cefotoxine described therein.

Tobacco

20   Tobacco leaf explants, roughly 5-10mm by 5-10mm, were cut from young leaves, approximately 3-5cm long and third to sixth from the apex of Nicotiniana tbabcum cv xanthi which were grown under axenic conditions in solid medium: Murashige Minimal Organics (#1118 Gibco Laboratories, New

25   York), 7% phytagar, 1mg/l indole-3-acetic acid, 0.15mg/l kinetin. The explants were plated on solid medium containing Murashige Minimal Organics, 6% phytagar, 40mg/l adenine sulfate, 2mg/l indole-3-acetic acid, 2mg/l kinetin. A sterile #1 Whatman filter paper (Whatman Ltd., Maidstone,

30   England) was placed on the top of the plate medium (explants are placed on top of filter) and they were incubated for 24 hours in the dark at 24°C.

The Agrobacterium were grown on AB medium (AB salts K_2HPO_4 3gm/l, NaH_2PO_4 H_2O 1.15g/l, NH_4Cl 1g/l, glucose 5g/l,

35   $FeSO_4$ 0.25mg/l, $MgSO_4$ 0.246mg/l, 0.14mg/l, 15g/l agar, 100 ug/l gentamycin sulfate and 100 ug/l streptomycin sulfate)

for 4-5 days. Single colonies were inoculated into 5mls of
MG/L broth (50% Luria broth and 50% mannitol-glutamate
salts medium (Garfinkel and Nester, *J.Bacteriol.*
(1980)144:732-743)) and were incubated overnight in a
5   shaker at 30°C and 180 R.P.M. before co-cultivation.

Following the preincubation period, the explants were
dipped into the bacterial suspension of 3.3 x 10^8 cells/ml
for approximately 5 minutes, blotted on sterile paper
towels and replated on the same plates. After 48 hours,
10   the explants were placed on selection medium containing the
same plate medium as above plus 350mg/l cefotaxime and
100mg/l kanamycin. The explants were transferred to fresh
media every 2 weeks. At the 6 week transfer thereafter,
shoot and green callus were trimmed from explants and
15   placed on solid media: Murashige Minimal Organics, .5mg/l
indole-3-acetic acid, 2 mg/l kinetin, 40mg/l adenine
sulfate, 350mg/l cefotaxime, 100mg/l kanamycin. Shoots
were harvested beginning about 4 weeks after co-cultivation
and placed in 50ml culture tubes with 25ml of solid medium
20   (7% bactagar 1mg/l indole-3-butyric acid, 350mg/l
cefotaxime, 100mg/l kanamycin) and grown at 24-28°C, 12
hours light, 12 hours dark, light intensity 80-100uEm^{-2}s^{-1}.
Shoots root in 1-2 weeks and were then transplanted into
soil and placed in growth chambers.

25

B.   Results

1.   Characterization of EF-1α Genomic Clone A (Le-
EF-A)

This sequence, comprising 4565 nucleotides in total is
30   presented in Figure 4. Notable features of the sequence
include:

a.   The EF-1α gene A has 2 introns (indicated
in small letters). One occurs in the 5' untranslated
region between nucleotides 43 and 44 of the cDNA (pCGN666).
35   This intron spans nucleotides 1680-2436 of this sequence.
The second intron occurs in the coding region between amino

acids 154 and 155. This intron spans nucleotides 2916-
2995.

b.   The 5' upstream region of the LeEF-A gene
is rather rich in A/T. The 2453 nucleotides upstream of

5   the initiation ATG are 75.1% A/T. When the intron within
this region (757 nucleotides) is examined, it is found to
be 70.4% A/T. This contrasts to the 1347 nucleotides in
the coding region (excluding intron 2) which shows 52.2%
A/T.

10

2.   <u>GUS Analysis of pCGN2157/pCGN2158 Transformed Plants</u>
Transformed tobacco plants were maintained <u>in vitro</u> on
media containing 0.7% Phytagar (Gibco), Murashige and Skoog
salts, 3% sucrose, 1 mg/l IAA, and 0.15 mg/l kinetin, pH

15   5.5. Transformed tomato plants were maintained <u>in vitro</u> on
media containing 0.65% Phytagar, Murashige and Skoog salts,
1x Nitsch vitaimins (Nitsch, C. and Nitsch, J.P. (1967)
Planta (Berl) <u>72</u>:355-370), 0.15 mg/l kinetin, pH 6.0.

a.   <u>Flurometric Analysis</u>

20   Analysis was performed on very young tobacco
shoot tips. Shoot tips, weighing approximately 50-100 mg
were quick frozen in liquid nitrogen and stored at -70° C.
until assayed. Samples were ground in 500 µl GUS
extraction buffer as described in Example II.7. In

25   addition, protein concentration was determined on a
separate aliquot using the Bio-Rad Protein Assay Kit (Bio-
Rad) with Bovine Serum Albumyn as standards. The data was
then converted to pmole MU/min/mg protein.

b.   <u>Localization of GUS Activity Within Tissue</u>

30   <u>In situ</u> GUS analysis was performed with X-
glucuronic acid (Molecular Probes, Inc., OR) as described
in (Jefferson, <u>Plant Mol. Biol. Reporter</u> (1987) <u>5</u>:387-405).
For the tobacco shoot tip, free hand sections approximately
10-20 cell layers thick were made and immersed in staining

35   solution overnight at 37°C. For tomato root tips, young
roots were immersed in staining solution overnight at 37°C.

54

3.   Expression Of EF-1α Gene A-GUS Chimera

To determine the pattern of expression of the EF-1α-
GUS chimera, in situ hybridizations were performed using X-
5   glucuronic acid. The action of B-glucuronidase on X-
glucuronic acid gives rise to a blue colored compound.

A young root tip from a pCGN2157 tomato plantlet and a
pCGN7001 (mas-GUS chimera - See Example II) plantlet were
examined and compared. In the pCGN7001 root tip,
10   development of color is seen throughout the root. In the
pCGN2157 root, the main color development is seen in the
very tip of the root. When endogenous EF-1α expression was
analyzed in tomato root tips by 35S - RNA in situ
hybridization (See, Example 1 and Pokalsy et al., (1989)
15   Nuc. Acids Res., 17:4661-4673) the majority of the
hybridization was seen in the very root tip (1st 1-2 mm).
The expression seen with pCGN2157 is similar and correlates
with the rapid growth that is taking place in the root tip.

A shot tip from a pCGN2157 tobacco plant was sectioned
20   and treated with X-glucuronic acid. The development of
color is seen in the very apex of the shoot, the
procambium, and the axillary buds. These regions are
characterized by rapidly dividing cells. This indicates
that the pattern for the EF-1α-GUS chimera correlates with
25   area of rapid cell division.

Levels of expression were analyzed by looking at GUS
levels in young shoot tips. Twenty pCGN2157 and twenty-
four pCGN2158 tobacco plants were analyzed (See, Figure 5).
Expression levels of tested events were compared to
30   different pCGN7001 tobacco plant events (mas-GUS chimera,
See, Example II) and against pCGN7320 repeated testing of a
clonal transformation event, clone pCGN7320 (double 35S-GUS
chimera). The "2157" and "2158" plants showed GUS levels
similar to or higher than the "d35S" plant and
35   significantly higher than the "mas plants." Mas is known
to be a relatively low level expresser (See, Example II)

while d35S promoters give relatively high level expression (Kay et al. (1987) Science, 236:1299-1302). The level of expression from observed pCGN2157 and pCGN2158 correlate well with the fact that EF-1α genomic clone A is high
5 expressing EF-1α gene.

It is evident from the above results, that plant cells and plants can be produced which have improved properties or may produce a desired product. In accordance with the
10 subject invention, rapidly dividing cells can provide for high levels of a desired product, which may impart resistance of the plant to a wide variety of environmental hazards, such as herbicides, pests, heat, drought, disease, other forms of stress, and the like. In addition, the rate
15 of growth of the plant may be modulated, so that higher or lower growth rates can be achieved, depending upon the particular situation. In this manner, crops may be grown more efficiently, in the substantial absence of weeds, may be less subject to attack by pathogens, so as to provide
20 for enhanced productivity and greater efficiency in utilization of land and chemical resources, and the like. The subject constructs provide for enhanced expression of the particular gene in rapidly dividing cells, so as to reduce the level of expression in those parts of the plant
25 where the expression product is not efficiently utilized. This provides for more efficient utilization of plant energy and resources, so that the energy and resources may be directed to a product of interest.

All publications and patent applications mentioned in
30 this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was
35 specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within
5   the scope of the appended claim.

WHAT IS CLAIMED IS:

    1.   A DNA sequence comprising:

    the 5' non-coding region obtainable from a plant elongation factor-1α gene comprising the transcriptional

5   and translational regulatory region and free of an intact elongation factor-1α gene coding region.

    2.   The DNA sequence according to Claim 1, where said 5' non-coding region is less than about 10kb.

    3.   The DNA sequence according to Claim 1, wherein

10   said sequence has fewer than 20 codons of said coding region.

    4.   The DNA sequence according to Claim 1, wherein said plant is tomato.

    5.   A DNA construct comprising in the direction of

15   transcription, a 5' non coding region obtainable from a plant elongation factor-1α gene comprising the transcriptional and translational regulatory region, a DNA sequence of interest other than a sense sequence of a plant elongation factor-1α gene and a 3' noncoding region

20   comprising transcriptional and translational termination regions functional in a plant cell, wherein expression of said DNA sequence of interest is regulated by said non coding regions.

    6.   The DNA construct according to Claim 5, wherein

25   said 3' non coding region is obtainable from a plant elongation factor 1-α gene.

    7.   The DNA construct according to Claim 5, wherein said DNA sequence of interest is a structural gene.

    8.   The DNA construct according to Claim 5, wherein

30   said DNA sequence of interest is an antisense sequence.

    9.   The DNA construct according to Claim 8, wherein said antisense sequence encodes a plant elongation factor-α or fragment thereof of at least about 15 nucleotides.

    10.   The DNA construct according to Claim 5, wherein

35   said plant is tomato.

58

11.   The DNA construct according to Claim 10, wherein
said DNA sequence encodes an enzyme.

12.   An expression cassette for integration into a
plant genome comprising:

5   in the direction of transcription, a transcriptional
and translational regulatory region obtainable from a plant
elongation factor-1α gene, a DNA sequence of interest other
than a sense sequence of a plant elongation factor-1α gene,
and transcriptional and translational termination regions

10   functional in said plant, wherein expression of said DNA
sequence of interest is regulated by said transcriptional
and translational regions.

13.   The expression cassette according to Claim 12,
further comprising

15   a marker for selection of transformed plant cells.

14.   The expression cassette according to Claim 12,
wherein said plant is tomato.

15.   The expression cassette according to Claim 12,
wherein said DNA sequence of interest is an antisense

20   sequence.

16.   The expression cassette according to Claim 15,
whrein said antisense sequence is a plant elongation
factor-1α gene or fragment thereof of at least about 15
nucleotides.

25   17.   An isolated cDNA sequence of from about 15
nucleotides to about 1.9kb encoding a plant elongation
factor-1alpha or fragment thereof.

18.   The isolated cDNA sequence according to Claim 17,
having the following sequence:

```
CTCCTCAGCTGTGCCGCATATCGCCTAATTTTTCTTCTCTAAGTTTCTTAGTGTCTCAAG ATG GGT AAA GAG AAG ATT CAC  81
                                                             M   G   K   E   K   I   H   7

ATC AGC ATT GTG GTC ATT GGT CAT GTT GAC TCT GGA AAG TCG ACT ACC ACT GGT CAC TTG ATC TAC AAG  150
I   S   I   V   V   I   G   H   V   D   S   G   K   S   T   T   T   G   H   L   I   Y   K   30

CTT GGT GGT ATT GAC AAG CGT GTT ATT GAG AGG TTC GAG AAG GAA CCT GCT GAG ATG AAC AAG AGG TCA  219
L   G   G   I   D   K   R   V   I   E   R   F   E   K   E   A   A   E   M   N   K   R   S   53

TTC AAG TAT GCC TGG GTG CTT GAC AAA CTT AAG GCA GAA CGT GAG CGT GGT ATC ACC ATT GAT ATT GCT  288
F   K   Y   A   W   V   L   D   K   L   K   A   E   R   E   R   G   I   T   I   D   I   A   76

TTG TGG AAG TTT GAG ACC ACT AAG TAC TAC TGC ACT GTT ATT GAT GCC CCC CGC CAC AGG GAT TTC ATC  357
L   W   K   F   E   T   T   K   Y   Y   C   T   V   I   D   A   P   G   H   R   D   F   I   99

AAG AAC ATG ATC ACT GGT ACC TCT CAG GCT GAC TGT GCT GTT CTC ATT ATT GAC TCC ACT ACT GGT GGT  426
K   N   M   I   T   G   T   S   Q   A   D   C   A   V   L   I   I   D   S   T   T   G   G   122
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TTT CAA CCT CGT ATC TCC AAA GAT CGT CAC ACC CGT CAA CAT CCA TTG CTT GCT TTC ACC CTT GGT GTC   495
 F   E   A   G   I   S   K   D   G   Q   T   R   E   H   A   L   L   A   F   T   L   G   V    145

AAG CAA ATG ATC TGC TGC TGT AAC AAG ATG GAT GCT ACC ACC CCC AAG TAC TCC AAG GCT ACG TAT GAT   564
 K   Q   M   I   C   C   C   N   K   M   D   A   T   T   P   K   Y   S   K   A   R   Y   D    168

GAA ATC GTG AAG GAA GTT TCT TCC TAC CTC AAG AAG GTT GGT TAC AAC CCT GAC AAA ATC CCC TTT GTT   633
 E   I   V   K   E   V   S   S   Y   L   K   K   V   G   Y   N   P   D   K   I   P   F   V    191

CCA ATC TCT GGT TTT GAA GGA GAC AAC ATG ATT GAG AGG TCT ACC AAC CTC GAC TGG TAC AAG GGA CCA   702
 P   I   S   G   F   E   G   D   N   M   I   E   R   S   T   N   L   D   W   Y   K   G   P    214

ACC CTC CTT GAG GCT CTT GAC CAG ATT AAC GAG CCC AAG AGG CCA TCA GAC AAA CCC CTC CGT CTT CCA   771
 T   L   L   E   A   L   D   Q   I   N   E   P   K   R   P   S   D   K   P   L   R   L   P    237

CTT CAG GAT GTT TAC AAG ATT GGT GGT ATT GGA ACT GTC CCT GTT GGT CGC GTT GAG ACT GGT GTG ATC   840
 L   Q   D   V   Y   K   I   G   G   I   G   T   V   P   V   G   R   V   E   T   G   V   I    260

AAG CCT GGT ATG GTT GTG ACC TTT GCC CCT ACT GGT TTG ACA ACT GAA GTC AAG TCT GTT GAG ATG CAC   909
 K   P   G   M   V   V   T   F   G   P   T   G   L   T   T   E   V   K   S   V   E   M   H    283

CAC GAA GCT CTC CAG GAG GCA CTC CCT GGT GAC AAT GTT GGG TTC AAT GTT AAG AAT GTT GCT GTT AAG   978
 H   E   A   L   Q   E   A   L   P   G   D   N   V   G   F   N   V   K   N   V   A   V   K    306

GAT CTT AAG CGT GGT TAT GTT GCC TCA AAC TCC AAG GAT GAC CCA GCC AAG GGG GCA GCC AGC TTC ACT   1047
 D   L   K   R   G   Y   V   A   S   N   S   K   D   D   P   A   K   G   A   A   S   F   T    329

GCC CAG GTC ATC ATC ATG AAC CAT CCT GGC CAG ATT GGA AAT GGA TAT GCT CCA GTG CTT GAT TGT CAC   1116
 A   Q   V   I   I   M   N   H   P   G   Q   I   G   N   G   Y   A   P   V   L   D   C   H    352

ACT TCC CAC ATT GCT GTC AAG TTT GCT GAG ATC TTG ACC AAG ATT GAC AGG CGT TCA GGT AAG GAA CTT   1185
 T   S   H   I   A   V   K   F   A   E   I   L   T   K   I   D   R   R   S   G   K   E   L    375

GAG AAG GAG CCT AAG TTC TTG AAG AAC GGT GAT GCT GGT ATG GTT AAG ATG ATT CCC ACC AAG CCC ATG   1254
 E   K   E   P   K   F   L   K   N   G   D   A   G   M   V   K   M   I   P   T   K   P   M    398

GTT GTT GAG ACC TTT GCT GAA TAC CCT CCA TTG GGT CGT TTT GCT GTG AGG GAC ATG AGG CAG ACT GTT   1323
 V   V   E   T   F   A   E   Y   P   P   L   G   R   F   A   V   R   D   M   R   Q   T   V    421

GCT GTT GGT GTT GTC AAG AAT GTT GAC AAG AAG GAC CCA ACT GGT GCC AAG GTC ACC AAG GCT GCC CAG   1392
 A   V   G   V   V   K   N   V   D   K   K   D   P   T   G   A   K   V   T   K   A   A   Q    444

AAG AAG GGG AAG TGA ACTGTGCAGTGTCATGCTGGTGTTTCAATGCTATTTATTTACATCGAGTGTGCTATCTAGACTCTGTTTT   1470
 K   K   G   K   .                                                                           448

ATTTAATTCTGCTTTAGTTTCGTCTTTCAGTTGTGTATTAGGTTGTGGTTCTCTCGACTTGTCTGTCAAGTATCCATTCTCAGAACTGGGTA   1569

CTTGATAGCCGGTCGCCAGCATGATTTTGAGTCTGTTATCATTTGCTTTTGTTGCAGGTGCCTTGGTTTGTTTTCCATGGTCGCTCGGGATT   1660

TTTGTTGCTTTAATTTCGAGATTTAAGTTTTCT-(POLY-A TAIL)
                   1692
```

19. An isolated genomic clone of from about 15nt to about 10kb nucleotides encoding a plant elongation factor-lalpha or fragment thereof.

20. An isolated genomic clone according to Claim 19, wherein said genomic clone is clone 61-1.

21. An isolated genomic clone according to Claim 19, wherein said genomic clone is clone A.

22. A plant cell comprising:

a DNA sequence comprising a 5'-non-coding region obtainable from a plant elongation factor-1α gene comprising the transcriptional and translational regulatory region and free of an intact elongation factor-1α gene coding region and a DNA sequence of interest other than a sense sequence of a plant elongation factor-1α gene, and transcriptional and translational termination regions

functional in said plant cell, wherein expression of said
DNA sequence of interest is regulated by said
transcriptional and translational regions.

23. The plant cell according to Claim 21, wherein
said DNA sequence is a structural gene.

24. The plant cell according to Claim 22, wherein
said DNA sequence of interest is an antisense sequence.

25. The plant cell according to Claim 22, wherein
said plant is tomato.

26. A plant comprising:
a plant cell according to any of Claims 21-23.

CTCCTCAGCTGTGCCGCATATCGCCTAATTTTTCTTCTCTAAGTTTCTTAGTGTCTCAAG ATG GGT AAA GAG AAG ATT CAC 81
                                        M G K E K I H 7

ATC AGC ATT GTG GTC ATT GGT CAT GTT GAC TCT GGA AAG TCG ACT ACC ACT GGT CAC TTG ATC TAC AAG 150
I S I V V I G H V D S G K S T T T G H L I Y K 30

CTT GGT GGT ATT GAC AAG CGT GTT ATT GAG AGG TTC GAG AAG GAA GCT GCT GAG ATG AAC AAG AGG TCA 219
L G G I D K R V I E R F E K E A A E M N K R S 53

TTC AAG TAT GCC TGG GTG CTT GAC AAA CTT AAG GCA GAA CGT GAG CGT GGT ATC ACC ATT GAT ATT GCT 288
F K Y A W V L D K L K A E R E R G I T I D I A 76

TTG TGG AAG TTT GAG ACC ACT AAG TAC TAC TGC ACT GTT ATT GAT GCC CCC GGC CAC AGG GAT TTC ATC 357
L W K F E T T K Y Y C T V I D A P G H R D F I 99

AAG AAC ATG ATC ACT GGT ACC TCT CAG GCT GAC TGT GCT GTT CTC ATT ATT GAC TCC ACT ACT GGT GGT 426
K N M I T G T S Q A D C A V L I I D S T T G G 122

TTT GAA GCT GGT ATC TCC AAA GAT GGT CAG ACC CGT GAA CAT GCA TTG CTT GCT TTC ACC CTT GGT GTC 495
F E A G I S K D G Q T R E H A L L A F T L G V 145

AAG CAA ATG ATC TGC TGC TGT AAC AAG ATG GAT GCT ACC ACC CCC AAG TAC TCC AAG GCT AGG TAT GAT 564
K Q M I C C C N K M D A T T P K Y S K A R Y D 168

GAA ATC GTG AAG GAA GTT TCT TCC TAC CTC AAG AAG GTT GGT TAC AAC CCT GAC AAA ATC CCC TTT GTT 633
E I V K E V S S Y L K K V G Y N P D K I P F V 191

CCA ATC TCT GGT TTT GAA GGA GAC AAC ATG ATT GAG AGG TCT ACC AAC CTC GAC TGG TAC AAG GGA CCA 702
P I S G F E G D N M I E R S T N L D W Y K G P 214

ACC CTC CTT GAG GCT CTT GAC CAG ATT AAC GAG CCC AAG AGG CCA TCA GAC AAA CCC CTC CGT CTT CCA 771
T L L E A L D Q I N E P K R P S D K P L R L P 237

CTT CAG GAT GTT TAC AAG ATT GGT GGT ATT GGA ACT GTC CCT GTT GGT CGC GTT GAG ACT GGT GTG ATC 840
L Q D V Y K I G G I G T V P V G R V E T G V I 260

AAG CCT GGT ATG GTT GTG ACC TTT GGC CCT ACT GGT TTG ACA ACT GAA GTC AAG TCT GTT GAG ATG CAC 909
K P G M V V T F G P T G L T T E V K S V E M H 283

CAC GAA GCT CTC CAG GAG GCA CTC CCT GGT GAC AAT GTT GGG TTC AAT GTT AAG AAT GTT GCT GTT AAG 978
H E A L Q E A L P G D N V G F N V K N V A V K 306

GAT CTT AAG CGT GGT TAT GTT GCC TCA AAC TCC AAG GAT GAC CCA GCC AAG GGG GCA GCC AGC TTC ACT 1047
D L K R G Y V A S N S K D D P A K G A A S F T 329

GCC CAG GTC ATC ATC ATG AAC CAT CCT GGC CAG ATT GGA AAT GGA TAT GCT CCA GTG CTT GAT TGT CAC 1116
A Q V I I M N H P G Q I G N G Y A P V L D C H 352

ACT TCC CAC ATT GCT GTC AAG TTT GCT GAG ATC TTG ACC AAG ATT GAC AGG CGT TCA GGT AAG GAA CTT 1185
T S H I A V K F A E I L T K I D R R S G K E L 375

GAG AAG GAG CCT AAG TTC TTG AAG AAC GGT GAT GCT GGT ATG GTT AAG ATG ATT CCC ACC AAG CCC ATG 1254
E K E P K F L K N G D A G M V K M I P T K P M 398

GTT GTT GAG ACC TTT GCT GAA TAC CCT CCA TTG GGT CGT TTT GCT GTG AGG GAC ATG AGG CAG ACT GTT 1323
V V E T F A E Y P P L G R F A V R D M R Q T V 421

GCT GTT GGT GTT GTC AAG AAT GTT GAC AAG AAG GAC CCA ACT GGT GCC AAG GTG ACC AAG GCT GCC CAG 1392
A V G V V K N V D K K D P T G A K V T K A A Q 444

AAG AAG GGG AAG TGA ACTGTGCAGTGTGATGCTGGTGTTTCGAATGCTATTTATTTACATCGAGTGTGCTATCTAGACTCTGTTTT 1478
K K G K .   448

ATTTAATTCTGCTTTAGTTTGGTCTTTGAGTTGTGTATTAGGTTGTGGTTCTCTGACTTGTCTGTCAAGTATCCATTCTCAGAACTGGGTA 1569

CTTGATAGGCGGTGGCGAGCATGATTTTGAGTCTGTTATCATTTGCTTTTGTTGAGGTGCCTTGGTTTGTTTTCCATGGTGGCTCGGGATT 1660

TTTGTTGCTTTAATTTGAGATTTAAGTTTTCT-(POLY-A TAIL) 1692

FIGURE 1

```
  1 MGKEKIHISIVVIGHVDSGKSTTTGHLIYKLGGIDKRVIERFEKEAAEMNKRSFKYAWVLDKLKAERERGIT 1
  1 MGKEKIHINIVVIGHVDSGKSTTTGHLIYKCGSIDKRTIEKFEKEAQEMGKGSFKYAWVLDKLKAERERGIT 2
  1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVLDKLKAERERGIT 3
  1 MGKEKTHVNVVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAELGKGSFKYAWVLDKLKAERERGIT 4
  1 MGKEKSHINVVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAELGKGSFKYAWVLDKLKAERERGIT 5

 73 IDIALWKFETTKYYCTVIDAPGHRDFIKNMITGTSQADCAVLIIDSTTGGFEAGISKDGQTREHALLAFTLG 1
 73 IDIALWKFETAKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGVGEFEAGISKNGQTREHALLAYTLG 2
 73 IDISLWKFETSKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGVGEFEAGISKNGQTREHALLAYTLG 3
 73 IDIALWKFETPKYNVTVIDAPGHRDFIKNMITGTSQADCAILIIAGGTGEFEAGISKDGQTREHALLAFTLG 4
 73 IDIALWKFETPKYQVTVIDAPGHRDFIKNMITGTSQADCAILIIAGGVGEFEAGISKDGQTREHALLAFTLG 5

145 VKQMICCCNKMDATTPKYSKARYDEIVKEVSSYLKKVGYNPDKIPFVPISGFEGDNMIERSTNLDWYKG 1
145 VKQLIVGVNKMDSTEPPFSEARFEEIKKEVSAYIKKFDYNEAAVAFVPISGWHGDNMLEASDRLHWYKGWNI 2
145 VKQLIVGVNKMDSTEPRYSQKRYEEIVKEVSTYIKKIGYNPDTVAFVPISGWNGDNMLEPSANMFWFKGWKV 3
145 FRQLIVAINKMDTTKWSQDRYNEIVKEVSGFIKKIGFNEKSVPFVPISGWHGDNMLDESTNMFWEFKGWNK 4
145 VRQLIVAVNKMDSVKWDESRFQEIVKEISNFIKKVGYNEKTVPFVPISGWNGDNMIEATTNAFWYKGWEK 5

214            PTLLEALDQINEFKRPSVKPLRLPLQDVYKIGGIGTVPVGRVETGVIKPGMVVTFGHTGLTTE 1
217 ERKEGKADGKTLIDALDAILPFSRFTEKPLRLPLQDVYKIGGIGTVPVGRVETGVLKPGMLVTFAFVNTTE 2
217 TRKDGNASGTTLLEALDCLLPFTRPTDKPLRLPLQDVYKIGGIGTVPVGRVETGILKAGMVVNFAEAAVTTE 3
215 ETKAGSKTGKTLLEAIDAIEPFVRPSDKPLRLPLQDVYKIGGIGTVPVGRVETGVIKPGMVVTFAPAGVTTE 4
215 ETKAGVVKGKTLLEAIDAIEQESRPTDKPLRLPLQDVYKIGGIGTVPVGRVETGVIKPGMVVTFAPAGVTTE 5

277 VKSVEMHHEAIQEALPGDNVGFNVKNVAVKDIKRGYVASNSKDDPAKGAASFTAQVIIMNHPGQIGNGYAPV 1
289 VKSVEMHHESLEQASPGDNVGFNVKNVSVKELRRGYVASDSKNNPARGSQDFEAQVIVLNHPGQISNGYIPV 2
289 VKSVENHHEAISEALPGDNVGFNVKNVSVKDVRRGNVAGDSKNDPPMEAAGFTAQVIILNHPGQISAGYAPV 3
287 VKSVEMHHETLTEGLPGDNVGFNVKNVSVKDIRRGNVCGDSKNDPAKESASFTAQVIILNHPGQISAGYSPV 4
287 VKSVEMHHEQLEQGVPGDNVGFNVKNVSVKEIRRGNVCGDAKNDPFKGCASFNATVJVLNHPGQISAGYSPV 5

349 LDCHTSHIAVKFAEIIKIDRRSGKELEKEPKFLKNGDAGMVKMIFIKPMVVETFAEYPPLGRFAVRVMRQT 1
361 LDCHTAHIACKFAEIKEKQDRRTGKTTEAEPKFIKSGDAAMITLVPSKPLCVEAFSDFPPLGRFAVRDMRQT 2
361 LDCHTAHIACKFAELKEKIDRRSGKKLEDGPKFLKSDEAAIVMVPGKPMCVESFSDYPPLGRFAVRDMRQT 3
359 LDCHTAHIACKFSELIEKIDRRSGKKMEDSPKFVKSGDSAIVKMVFSKPMCVEAYTDYPPLGRFAVRDMRQT 4
359 LDCHTAHIACREDELLFKNDRRSGKKLEDHPKFLKSGDAAIVKFVFSKPMCVEAFSEYPPLGRFAVRDMRQT 5

421 VAVGVMKNVDKKDPTGAKVTKAAQKKGK 1
433 VAVGVIKSVNEKDPTAGKVTKAAEKAGKKK 2
433 VAVGVIKAVDKKAAGAGKVTKSAQKAQKAK 3
431 VAVGVIKAVEKVDKAGKVTKAAAKASKK 4
431 VAVGVIKSVDKTEKAAKVTKAAQKAAKK 5
```

FIGURE 2

1   TATTATTATAAAAAAAAAAAAACTACCGACTAAGCTCATTCATCCTCCTCTATATATATTACCAATAACC   69

70   TCTTGTAAACTTAAATAAAGTTTCCTTCATTTTTCAACTTAATATATGTGCTGTGCCTTCTCTAGGTTA   138

139   GATATATATATTGATATTCCATCACTCATTTTTGCTTATTTTATGTAAAATTTCATGCAAATTTGTTCC   207

208   ATGATCCATCAGTTTTTAATAAAAATTGATACTAGATAGTGTAATTTGATGCAAATTTGGTTGATGTTTG   276

277   CTGAGTTTATATATAATTAATAGGCTGTTTGATCCGTTTCATTTTATATATCTTTTTCTTTTTCGTCTG   345

346   TCTCAAAAAAGATGTCACTTTATTATGAGAAATAATTACTTAAAAACTTCATTTTTACACGTAATGAAT   414

<pre>
                                                          DraI
                                                           |
415 TGATTTATTGCTACATAAATATTTAAGTATTGCTTTGAACTATAAATATCGTTCTTTTAAATATTATGT 483
                                                          473
</pre>

<pre>
                              ScaI
                               |
484 CAAGTCAAAATTGTGTCACGTGAAATGAGACAGAGTGAGTACTGGTTTTTATTTGTTTGCTTTCTGTTT 552
                                                524
</pre>

553   CTATATATTTGATAGTAGCTTGGATTAATTTAACTCAAGATTTGTGACTAAAGTTCATTAGTTTTGTTC   621

<pre>
                                     XbaI
                                      |
622 ATCTTAAATGTCTCATAATTCCTGGAGAAGGATGCTTGCCTTCTAGATTGTTCAGTCTGTGTATTGTTT 690
                                          664
</pre>

<pre>
    BclI
     |
691 GATCATTGTTGGATGATCTGTTTCGTTGATTGGCTACCTAAACTCGATTTTGATGTATTCGATTTTTCA 759
691
</pre>

<pre>
                                            AccI
                                             |
760 GAATTTCTCTATGTTTTGTGTGAATATCAGATTGTTGTAGACATAGAACACAACTTTTAGATGTATTTG 828
                                            798
</pre>

<pre>
              HincII   BclI
                | |
829 ACATTTGTTTCTATTGTTGACAAGACTTCTAGTTGATCAACATGGGTAAAGAGAAGGTTCACATCAACA 897
              844   863   METGlyLysGluLysValHisIleAsnI
</pre>

<pre>
             HincII
             BalI
              | |
898 TTGTGGTTATTGGCCATGTTGACTCTGGCAAATCGACTACCACTGGTCACTTGATCTACAAGCTAGGTG 966
     leValValIleGlyHisValAspSerGlyLysSerThrThrThrGlyHisLeuIleTyrLysLeuGlyG
             911 915
</pre>

<pre>
        ClaI
         |
967 GTATCGATAAACGTGTCATCGAGAGGTTTGAGAAAGAAGCTGCTGAGATGAACAAGAGGTCATTCAAGT 1035
     lyIleAspLysArgValIleGluArgPheGluLysGluAlaAlaGluMETAsnLysArgSerPheLysT
        971
</pre>

<pre>
1036 ATGCCTGGGTTCTTGACAAGCTCAAGGCTGAACGTGAACGCGGTATCACTATTGATATTGCCTTGTGGA 1104
     yrAlaTrpValLeuAspLysLeuLysAlaGluArgGluArgGlyIleThrIleAspIleAlaLeuTrpL
</pre>

FIGURE 3
Page 1 of 4

```
                         ScaI
                          |
1105 AGTTTGAGACTACCAAGTACTATTGTACTGTCATTGATGCTCCTGGTCATAGGGATTTCATCAAGAACA 1173
     ysPheGluThrThrLysTyrTyrCysThrValIleAspAlaProGlyHisArgAspPheIleLysAsnM
                                                                  1123
                    KpnI
                     |
1174 TGATTACTGGTACCTCTCAAGCTGACTGTGCTGTCCTGATTATCGACTCCACGACTGGTGGTTTTGAAG 1242
     ETIleThrGlyThrSerGlnAlaAspCysAlaValLeuIleIleAspSerThrThrGlyGlyPheGluA
                   1187


1243 CTGGTATCTCTAAGGATGGACAGACTCGTGAACATGCGTTGCTTGCTTTCACACTTGGTGTTAGGCAAA 1311
     ·laGlyIleSerLysAspGlyGlnThrArgGluHisAlaLeuLeuAlaPheThrLeuGlyValArgGlnM

1312 TGATTTGTTGCTGCAACAAGGTTTGCTCTTTCTTCAAGTTTTAACTCACTCTAACACCCCCTTAACACC 1380
     ETIleCysCysCysAsnLys

1381 CCAATGCCTAAAACGGAGATGGACCTGACTCTGATACCATGTAAAGATATGACATCTGGACCTAACTCA 1449

1450 ACCCAAAAGCTAACTCATGAGCTACGAGGGGAGGATTGTTCAAGTCCATTTCCCAATGATTAGCTAACT 1518

1519 CATGAGGGTAGCTCATGAGGGGTGGATTGTCCAAGTTCATTCTCTAATGATTGGGACTTTAACCAACTC 1587

1588 AAACAGATGTTACCTGAAGAATGCTGCTTCATGTTTTGCTAATGTGTTTTTACTCCTTGTGTAGATGGA 1656
                                                                     METAs
                         ScaI·
                          |
1657 TGCTACCACACCTAAGTACTCCAAGGCTAGGTATGATGAAATTGTGAAGGAAGTGTCTTCTTACCTTAA 1725
     pAlaThrThrProLysTyrSerLysAlaArgTyrAspGluIleValLysGluValSerSerTyrLeuLy
                                                             1674

1726 GAAGGTTGGTTACAACCCTGACAAGATCCCATTTGTGCCGATTTCTGGTTTCGAAGGAGATAATATGAT 1794
     sLysValGlyTyrAsnProAspLysIleProPheValProIleSerGlyPheGluGlyAspAsnMETIl

1795 TGAGAGATCAACTAACCTGGACTGGTACAAGGGTCCAACCCTTCTTGAGGCTCTTGACCAGCTTAATGA 1863
     eGluArgSerThrAsnLeuAspTrpTyrLysGlyProThrLeuLeuGluAlaLeuAspGlnLeuAsnGl

1864 GCCTAAGAGGCCCTCGGACAAACCCTTGCGTCTTCCACTTCAGGATGTCTATAAGATTGGGGGGATTGG 1932
     uProLysArgProSerAspLysProLeuArgLeuProLeuGlnAspValTyrLysIleGlyGlyIleGl

1933 GACTGTTCCTGTTGGTCGTGTTGAGACTGGTGTGATTAAGCCTGGTATGGTTGTGACTTTTGGTCCTAC 2001
     yThrValProValGlyArgValGluThrGlyValIleLysProGlyMETValValThrPheGlyProTh
                              NsiI
                               |
2002 TGGTCTGACTACTGAAGTTAAGTCTGTTGAGATGCATCATGAAGCTCTTCAAGAGGCACTGCCTGGTGA 2070
     rGlyLeuThrThrGluValLysSerValGluMETHisHisGluAlaLeuGlnGluAlaLeuProGlyAs
                                            2038


2071 TAATGTTGGTTTTAACGTCAAGAATGTTGCGGTTAAGGATCTTAAACGTGGATTTGTTGCTTCCAACTC 2139
     pAsnValGlyPheAsnValLysAsnValAlaValLysAspLeuLysArgGlyPheValAlaSerAsnSe


2140 CAAGGATGACCCAGCTAAGGGGGCTGCTAGCTTCACCGCTCAGGTCATCATCATGAACCATCCAGGACA 2208
     rLysAspAspProAlaLysGlyAlaAlaSerPheThrAlaGlnValIleIleMETAsnHisProGlyGl
```

<p style="text-align:center">FIGURE 3
Page 2 of 4</p>

```
                                                                 BglII
                                                                  |
2209 GATTGGAAATGGATATGCTCCAGTGCTGGACTGTCACACCTCCCATATTGCTGTCAAGTTTGCTGAGAT 2277
     nIleGlyAsnGlyTyrAlaProValLeuAspCysHisThrSerHisIleAlaValLysPheAlaGluIl
                                                                       2275

                                   XhoI
                                   AvaI
                                    |
2278 CTTGACTAAGATTGACAGGCGTTCTGGTAAGGAACTCGAGAAGGAGCCTAAGTTCTTGAAGAACGGTGA 2346
     eLeuThrLysIleAspArgArgSerGlyLysGluLeuGluLysGluProLysPheLeuLysAsnGlyAs
                                   2313

                         NcoI
                          |
2347 TGCTGGTATGGTTAAGATGATTCCCACCAAGCCCATGGTTGTTGAGACATTCTCCGAGTACCCACCATT 2415
     pAlaGlyMETValLysMETIleProThrLysProMETValValGluThrPheSerGluTyrProProLe
                         2380

                                                   HincII
                                                    |
2416 GGGACGTTTTGCTGTGAGGGACATGCGTCAAACTGTTGCTGTTGGTGTTATCAAGAATGTTGACAAGAA 2484
     uGlyArgPheAlaValArgAspMETArgGlnThrValAlaValGlyValIleLysAsnValAspLysLy
                                                   2474


2485 GGACCCATCTGGAGCCAAGGTCACCAAGGCTGCTCAGAAGAAAAAATGAATAGTGCAGTTCAACTCTGG 2553
     sAspProSerGlyAlaLysValThrLysAlaAlaGlnLysLysLys

              EcoRV
               |
2554 AGTTACCAGAGATATCAGTATCTACTATAATAAAGTGTGTTATGAAAACTTGCTATACTTTACTCTTCC 2622
              2567

2623 TGCTCATGTTCAGTCTTTTATGTTGTTGTTTGGTTCTTGTTTTCTGCCCTGTATGTCAGGTATCCTTTC 2691

2692 CCAGAACTGGGTGCTCGACAGACGGTGGCAAAAGACACAGTTAAGAGTCATTTGGTTTATCTTTTGAGG 2760

              HindIII
               |
2761 TGCCTAGGTTCATCCAAGACAGGCTAAGAAGCTTTGTTATTTATGTTTTCTTATAACACGTAAATCGTA 2829
              2790

2830 CTTTTTCTGTTTTTGTGCTGATAATCTTCTATTGCATCATCTATCCTGTTTTTCCCTTGACATAGTTTG 2898

2899 GATAACTTGTTTGAAGTCTCTGGATGAGGTTGTGGGCAACTCAACTATTTCATTGAATACTTGCTACCT 2967

              HindIII
               |
2968 CCCGTCAATATGAATACCAATTAACTCCGCCCAGCAAAGCTTAGACAAATGAGAAAAGATTACCTAGCA 3036
              3005

     BclI
      |
3037 TTATGATCATCACTAATAATCAAGGTCACTTAGGAACACAACATCTCTCTGATAAATACTATAAGACAA 3105
     3041
```

FIGURE 3
Page 3 of 4

3106 ATTGAGGTTTCCCATTGAATCAGGCAACAACCTAATAGGTAGCTACAAATACACAATTTTAGGGTTCTG 317

                            Acc
                            |

3175 GAGGGGGACAGTCATCAGGTGCATGTCGTATACTCTAAAAGTGGTAAGTAGTGCATACTTAGAGAGCCG 3243
                         3204

3244 ATATGGGTACAGCAACATTTTGGACAGTCCGAGCAACATAAATTTACTCCCACTATCCCACACTGTATA 3312

                                                   EcoRI
                                                  |

3313 AAAGCTATGAACCAAGAAACTGAACCAAAAGTTGCTAGTAATATAGTATTTATCTTGAATTC 3374
                                                          3370

FIGURE 3
Page 4 of 4

EF-GENOMIC   Linear   LENGTH = 4565

```
                  DdeI                           Sau3AI
                   |                               |
   1 ATGTGTGTTATATTAGGTCTTAGACGAAATTCATTCCCAAAAGGTAGATCAAATAGAGGAGGATTGTCC   69
                  20                              47
```

```
                                        TaqI
                                        ClaI
                                         |
  70 AAATATTATAAGAAGTCACTCATTTCATTAATCATCGATGTAGAATTTTTTTCATTCTTTAACACCCTA   138
                                        105
                                        105
```

```
                  DdeI
                   |
 139 TATCACGTCAAGTGCTTAGCATGTTGTGTATGGACAATTTTGACTTTAAGAGCCCCAACATTGCACACT   207
                  154
```

```
 208 TTACCTTCTTTTTTTTTTGAAAAAATTAACAACAATTTTTTTTTCCTACTTATTCAATACCTTGTTCAAT   276
```

```
                  DdeI      RsaI
                   |         |
 277 AATAAAGAACTAAGATAGTACGAAGAGAATTTTATTATTTTGTCTAGCAATTAATAATAATAAATAATA   345
                  287       296
```

```
 346 ATGTAACAAACATTTCTATGAAATATATATTATTCTCTTTTCCAAGCAAAATTAAAAAATGATAACCAA   414
```

```
          DraI                                     AluI
           |                                        |
 415 CAATTTAAAAACTTATCCTACAATATAAGTTCATAAAAAAATAATTAGCTTGCCAATTGGCTTAACCAG   483
          421                                      463
```

```
 484 ATTGCAATTATATATATATATATAATAATAATAATAATAATAATAATAATAAATATTTTTAACTTTTTC   552
```

```
                                        DraI
                                         |
 553 TCAACAAAAAAAATACTGTTGCTGCTTTCAGTTTAAATCAGTTTTAAGAGTTTACGAAAAATTTAATTA   621
                                        587
```

```
          DraI
           |
 622 TTTAAAGTAAGTATATTTTTCAATAACAATGCCAAAACCTCTAATATTCTTAAATTTAACAATTGCCAA   690
          625
```

```
 691 GTGATTTTTAGTTAGTTTGCTATTACTATTGTTGTTATCCTTACTACTAGCATTATTACTATTAATTAA   759
```

FIGURE 4
Page 1 of 8

760 TTATTATTATTATCATCACTATCATTATTAGTAAAAGGTTTTTTTTATGTTTGTATCTTTTAATTTAAT 828

```
         DraI                            DraI
          |                               |
829 ATTTAAATTAAATACATTTACATTTATTTTGCAATATTTAAAAATGATTAGTTTGTCAAATAGTAGAGT 897
       833                              . 868
```

```
                                        TaqI
                                        SalI
                                        HincII
         DraI                           AluI AccI
          |                              |   |||
898 TCATTTAAAATTCTTCAGCCATATAGTTCTATTTTTAAGCTAGTCGACTTTTTTTTTTCTTACTGAAAAT 966
       904                             937 942
                                           940
                                           941
                                           942
```

967 TAATATTTTTTTCTTTTTGAAATACTAATACATCTAAATTTAACAATTGCCAAAGTGATTTTTAATTAG 1035

```
     AluI
      |
1036 CTTGCTGGCTAATCACAATACAAATTACTCTCCTTTACTATATAAGTAAATTTTTATTGCTATATTTGT 1104
    1036
```

```
                                                          DraI
                                                           |
1105 TATTATTATTATTATTAATATTTATTTTCTACAAATTTAACAATATTTTATTTTATATCATTTTAAAAA 1173
                                                          1169
```

```
           EcoRI          EcoRI                          AluI
            |              |                              |
1174 GATAAGTAATGAAATATTAAGAATTCGTTTAGAATTCTTTTGCAGGTGGGTTTCTATTTGTAAGCTAAT 1242
           1195           1206                           1238
```

```
                                        AluI          DraI
                                         |             |
1243 CTTTTTCAGTTATCTTTTTTTTTTAATCTTTATTATTATTATAGCTATATCTTTTTCTTTTAAAATTAA 1311
                                        1287          1304
```

1312 CATTATCTATTAAAGATAATTTCAATAAAAGAGTAAAAATTAATTTAGAGTTCTACTTTCTTCAAATTT 1380

```
     DraI       DraI
      |          |
1381 CTATTTTAAAAAATACTTTTAAAACTTGATGTATTTTTTTACGTGGTTTTTCACTATGACTTGATTTCT 1449
       1388      1401
```

FIGURE 4
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1450 GTTTTATTATAATATGTATAAATATAAAAATAGATATTCCATAACATATTATAAAAAATGTAAGGGGCA 1518

1519 TTTACGTAAATAGATAGACTTAAAAGAGGCACCGAGTGAACCCTAATTCTCATCGTTGAGACTATAAAA 1587

```
                                                         PvuII
                                                   DdeI
                                 START OF cDNA     AluI
                                    |    |    |
1588 TGCCCATTATCCCATTCGCATTGTCTCTTCATTACTTTTGCTGTGATTTCTCCTCAGCTGTGCCGCATA 1656
                                              1645
                                         1641
                                              1645


                                                   TaqI
                                                   HinfI
                      DdeI                         |  |
                      |
1657 TCGCCTAATTTTTCTTCTCTAAGgtttcatcatcttccaattttctttaatctcgattcaatttttta 1725
                 1676                                  1713
                                                  1711


           Sau3AI
           |
1726 tgtttgatctgttattgttctgtcactacatgtgtttttcagttgttttactagatgattttcactgtc 1794
        1731


            Sau3AI
            |
1795 ttcttgttagatcatacatatattgaaaatgttttggattgacttttttgtattgtgaatatctgttat 1863
        1804


                           Sau3AI         AluI
                           |              |
1864 tgtttgatagttgttcagtatttacacagccgatctgtgttatgagcttggtcataactatttctctgt 1932
                         1895             1910


             Sau3AI
             BglII                  NdeI
             |                      |
1933 atgtaaatacagatctgttaatgtttgtaatcaattttttcatatgcactgttgatattgttctctctcc 2001
          1944                   1974


            HinfI
            |
2002 tgtcctgttatatgttgatatgattcggttttttgtataacttgaactaaacactagtcctaaatgtttt 2070
          2024


                                                      HindIII
                                                      AluI
                                                      |  |
2071 ttttactatttaagatttatataatatggatagatttgttgagttcctagtctctgaagaggttaagct 2139
                                                         2138
                                                         2136
```

<div align="center">

FIGURE 4
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</div>

2140 tgctgtagttgtttaccagttgagttgcaatactaaaaatcaattcaattactgatattttttgctgtt 2208

```
                          ScaI
                          RsaI                                      EcoRI
                          |                                         |
2209 taggtttttgacgaagtactttaatttgctttattgaactaaaaacgtagtcctgaattcattgcaagt 2277
                          2226                                      2264
                          2226
```

```
                  AluI                                                   AluI
                  |                                                      |
2278 gtgaaagctatagttcattgttttttgttgcaattcttgaaaaatcaattggtcaagctataatggattt 2346
                  2285                                                   2334
```

```
                  DraI          HhaI
                  |             |
2347 acttttttctgttttaaatgttgaatgcgctgaatttatgaatgggttgcatggttttttgaaatatgttg 2415
                  2361          2375
```

```
                          DdeI                    HinfI
                          |                       |
2416 ttgtgtgttgtgtaaatgcagTTTCTTAGTGTCTCAAGATGGGTAAAGAGAAGATTCACATCAGCATTG 2484
                                              METGlyLysGluLysIleHisIleSerIleV
                          2441                                      2469
```

```
                                  TaqI
                                  SalI
                  HinfI           HincII                         HindIII
          HincII                  AccI            Sau3AI          AluI
          |     |                 |||             |               | |
2485 TGGTCATTGGTCATGTTGACTCTGGAAAGTCGACTACCACTGGTCACTTGATCTACAAGCTTGGTGGTA 2553
     alValIleGlyHisValAspSerGlyLysSerThrThrThrGlyHisLeuIleTyrLysLeuGlyGlyI
          2499                    2515            2534            2544
                  2503            2513                            2542
                                  2514
                                  2515
```

```
                                  DdeI
                  TaqI            AluI
                  |               | |
2554 TTGACAAGCGTGTTATTGAGAGGTTCGAGAAGGAAGCTGCTGAGATGAACAAGAGGTCATTCAAGTATG 2622
     leAspLysArgValIleGluArgPheGluLysGluAlaAlaGluMETAsnLysArgSerPheLysTyrA
                  2579         2590
                                  2594
```

```
     BstNI
     |
2623 CCTGGGTGCTTGACAAACTTAAGGCAGAACGTGAGCGTGGTATCACCATTGATATTGCTTTGTGGAAGT 2691
     laTrpValLeuAspLysLeuLysAlaGluArgGluArgGlyIleThrIleAspIleAlaLeuTrpLysP
     2625
```

FIGURE 4
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```
                                        MspI
                                        HpaII                       Sau3AI
              ScaI                      HaeIII                      BclI
          DdeI RsaI                    | |                          |
          |    |                       | |                          |
2692 TTGAGACCACTAAGTACTACTGCACTGTTATTGATGCCCCCGGCCACAGGGATTTCATCAAGAACATGA 2760
     heGluThrThrLysTyrTyrCysThrValIleAspAlaProGlyHisArgAspPheIleLysAsnMETI
        2702 2707                        2735                            2759
             2707                        2732                            2759
```

```
          KpnI
          RsaI DdeI                            HinfI                  AluI
          | |  |                               |                      |
2761 TCACTGGTACCTCTCAGGCTGACTGTGCTGTTCTCATTATTGACTCCACTACTGGTGGTTTTGAAGCTG 2829
     leThrGlyThrSerGlnAlaAspCysAlaValLeuIleIleAspSerThrThrGlyGlyPheGluAlaG
        2769 2774                              2803                     2827
             2771
```

```
                                    NsiI                               Sau3AI
                                    |                                  |
2830 GTATCTCCAAAGATGGTCAGACCCGTGAACATGCATTGCTTGCTTTCACCCTTGGTGTCAAGCAAATGA 2898
     lyIleSerLysAspGlyGlnThrArgGluHisAlaLeuLeuAlaPheThrLeuGlyValLysGlnMETI
                                    2865                               2897
```

```
                                                              RsaI
                                                         AluI
                                                         |    |
2899 TCTGCTGCTGTAACAAGgtttgctttaattcgtagatggaattaattgttaatttaagctgtacatgtc 2967
     leCysCysCysAsnLys                                   2957
                                                              2961
```

```
                                              ScaI
                                              RsaI
                                              |
2968 atgctaattctgttattattttttgtagATGGATGCTACCACCCCCAAGTACTCCAAGGCTAGGTATGA 3036
                                 METAspAlaThrThrProLysTyrSerLysAlaArgTyrAs
                                              3018
```

```
3037 TGAAATCGTGAAGGAAGTTTCTTCCTACCTCAAGAAGGTTGGTTACAACCCTGACAAAATCCCCTTTGT 3105
     pGluIleValLysGluValSerSerTyrLeuLysLysValGlyTyrAsnProAspLysIleProPheVa
```

```
                                    AccI        TaqI      RsaI  AvaII
                                    |           |         |     |
3106 TCCAATCTCTGGTTTTGAAGGAGACAACATGATTGAGAGGTCTACCAACCTCGACTGGTACAAGGGACC 3174
     lProIleSerGlyPheGluGlyAspAsnMETIleGluArgSerThrAsnLeuAspTrpTyrLysGlyPr
                                    3147        3157      3165  3171
```

```
                                  HaeIII
                                  |
3175 AACCCTCCTTGAGGCTCTTGACCAGATTAACGAGCCCAAGAGGCCATCAGACAAACCCCTCCGTCTTCC 3243
     oThrLeuLeuGluAlaLeuAspGlnIleAsnGluProLysArgProSerAspLysProLeuArgLeuPr
                                  3218
```

FIGURE 4
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                                                              Sau3AI
                                                              BclI
                                                                I
3244 ACTTCAGGATGTTTACAAGATTGGTGGTATTGGAACTGTCCCTGTTGGTCGCGTTGAGACTGGTGTGAT 3312
      oLeuGlnAspValTyrLysIleGlyGlyIleGlyThrValProValGlyArgValGluThrGlyValIl
                                                                         3310

        BstNI   HaeIII
          I   I
3313 CAAGCCTGGTATGGTTGTGACCTTTGGCCCTACTGGTTTGACAACTGAAGTCAAGTCTGTTGAGATGCA 3381
      eLysProGlyMETValValThrPheGlyProThrGlyLeuThrThrGluValLysSerValGluMETHi
              3319   3340

        AluI BstNI   BstNI
          I   I   I
3382 CCACGAAGCTCTCCAGGAGGCACTCCCTGGTGACAATGTTGGGGTTCAATGTTAAGAATGTTGCTGTTAA 3450
      sHisGluAlaLeuGlnGluAlaLeuProGlyAspAsnValGlyPheAsnValLysAsnValAlaValLy
              3390 3396   3409

                                                              AluI
                                                                I
    Sau3AI   I
      I
3451 GGATCTTAAGCGTGGTTATGTTGCCTCAAACTCCAAGGATGACCCAGCCAAGGGGGCAGCCAGCTTCAC 3519
      sAspLeuLysArgGlyTyrValAlaSerAsnSerLysAspAspProAlaLysGlyAlaAlaSerPheTh
      3452   3514

                            HaeIII
                            BstNI
        BstNI   BalI
          I   I I
3520 TGCCCAGGTCATCATCATGAACCATCCTGGCCAGATTGGAAATGGATATGCTCCAGTGCTTGATTGTCA 3588
      rAlaGlnValIleIleMETAsnHisProGlyGlnIleGlyAsnGlyTyrAlaProValLeuAspCysHi
        3525   3550
                            3547
                            3550

                          Sau3AI
                          DdeI
                            BglII
                          I I
3589 CACTTCCCACATTGCTGTCAAGTTTGCTGAGATCTTGACCAAGATTGACAGGCGTTCAGGTAAGGAACT 3657
      sThrSerHisIleAlaValLysPheAlaGluIleLeuThrLysIleAspArgArgSerGlyLysGluLe
                            3619
                          3616
                            3619

                                              HinfI   NcoI
          DdeI   I   I
3658 TGAGAAGGAGCCTAAGTTCTTGAAGAACGGTGATGCTGGTATGGTTAAGATGATTCCCACCAAGCCCAT 3726
      uGluLysGluProLysPheLeuLysAsnGlyAspAlaGlyMETValLysMETIleProThrLysProME
              3670   3710   3724
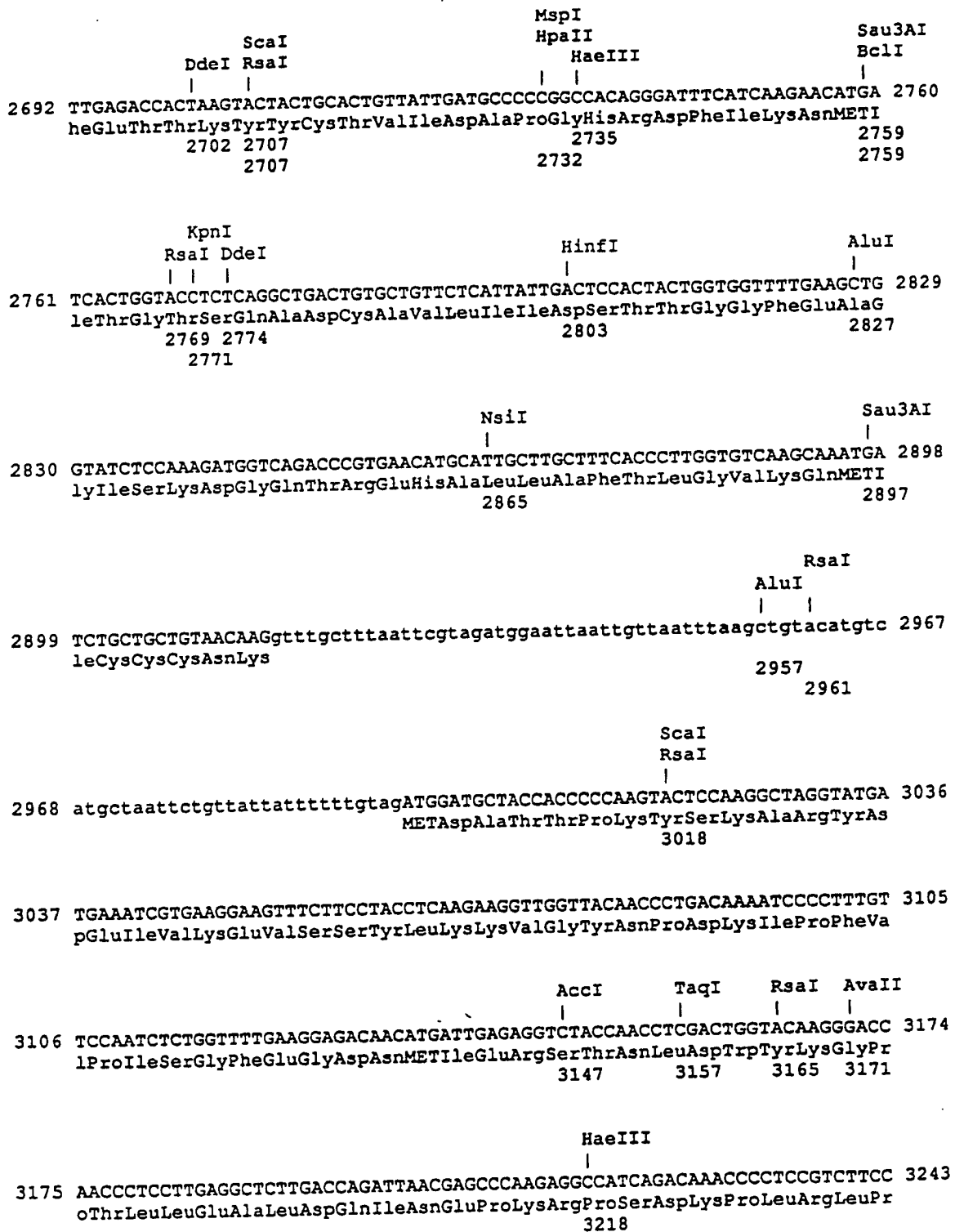

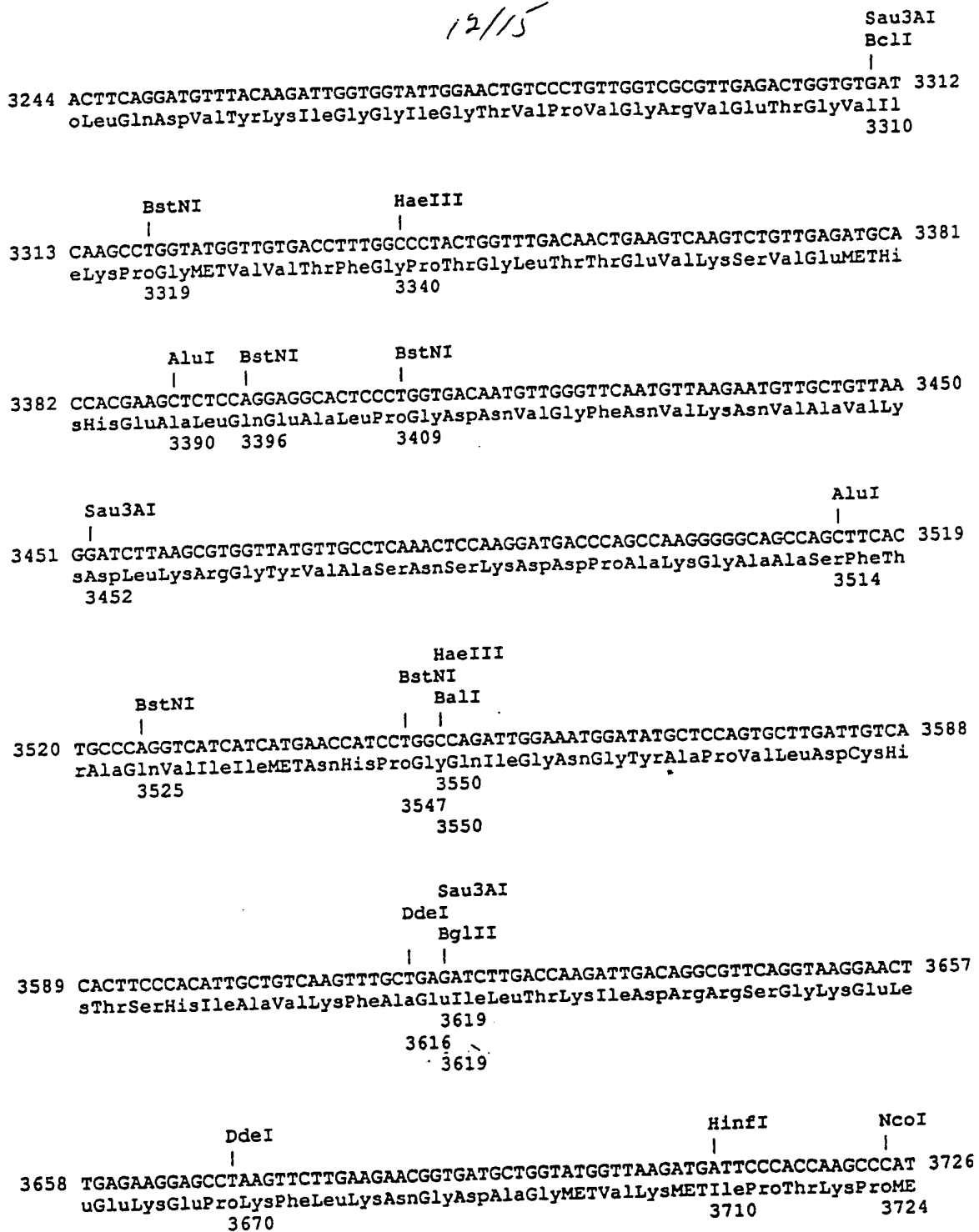   FIGURE 4
                           Page 6 of 8

```
3727 GGTTGTTGAGACCTTTGCTGAATACCCTCCATTGGGTCGTTTTGCTGTGAGGGACATGAGGCAGACTGT 3795
     TValValGluThrPheAlaGluTyrProProLeuGlyArgPheAlaValArgAspMETArgGlnThrVa
```

```
                      HincII        AvaII                    BstEII
                        |             |                        |
3796 TGCTGTTGGTGTTGTCAAGAATGTTGACAAGAAGGACCCAACTGGTGCCAAGGTGACCAAGGCTGCCCA 3864
     lAlaValGlyValValLysAsnValAspLysLysAspProThrGlyAlaLysValThrLysAlaAlaGl
                        3818          3830                    3848
```

```
                                             TaqI                  TaqI
                                              |                     |
3865 GAAGAAGGGGAAGTGAACTGTGCAGTGTGATGCTGGTGTTTCGAATGCTATTTATTTACATCGAGTGTG 3933
     nLysLysGlyLys .                          3906                  3926
```

```
        XbaI
          HinfI                 ,
        |  |
3934 CTATCTAGACTCTGTTTTATTTAATTCTGCTTTAGTTTGGTCTTTGAGTTGTGTATTAGGTTGTGGTTC 4002
           3942
        3938
```

```
                          DdeI      RsaI
                           |         |
4003 TCTGACTTGTCTGTCAAGTATCCATTCTCAGAACTGGGTACTTGATAGGCGGTGGCGAGCATGATTTTG 4071
                           4030      4042
```

```
        HinfI                              NcoI    AvaI
        |                                   |       |
4072 AGTCTGTTATCATTTGCTTTTGTTGAGGTGCCTTGGTTTGTTTTCCATGGTGGCTCGGGATTTTTGTTG 4140
     4072                                  4117    4126
```

```
            End of cDNA                        HincII
                 |                               |
4141 CTTTAATTTGAGATTTAAGTTTTCTACTCATTACGTTACATTTAAGTTAACATGTTACCTTTGCTAAAG 4209
                                                 4186
```

```
                   DdeI
                   AluI
                   ||
4210 GGTGGTGATTTGGTTATTAATAGCTCAGTTGTATAATGGTGCATCAAATTCTGCCTGTAGGATTTGTTG 4278
                   4233
                   4234
```

```
                                                        RsaI
                                                         |
4279 CAATTTTTATGCTACTGTGCTGACTTCCTAATGCACTATAACATGAGTGATTTTGTACATGGTAACTTG 4347
                                                         4335
```

FIGURE 4
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```
                                              HincII
                                              BstNI
                                               | |
     4348 AAATGGTGAATTGAGGCAGAGTGACAACCAAGTCGCCTGGTCAACCCCGCACTGGGGATGACAACCATG 4416
                                              4385
                                                4387


                RsaI         TaqI
                 |            |
     4417 TGATTTTGTACGTGATAATTCGAAATGGTGAAGTGAGGTAGAACTTGCACCAGATGGCAGCCAGTGTAT 4485
                4426 ·       4437


     4486 TCATAAAACCATATCAAAGTTGCTATGAACATTTTACTAATTCTCATTATTCTTGATTTTTGATGAGAT 4554


                Sau3AI
                 |
     4555 GGAGAAGATCC 4565
                4561
```

<div align="center">

FIGURE 4
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</div>

/5//5
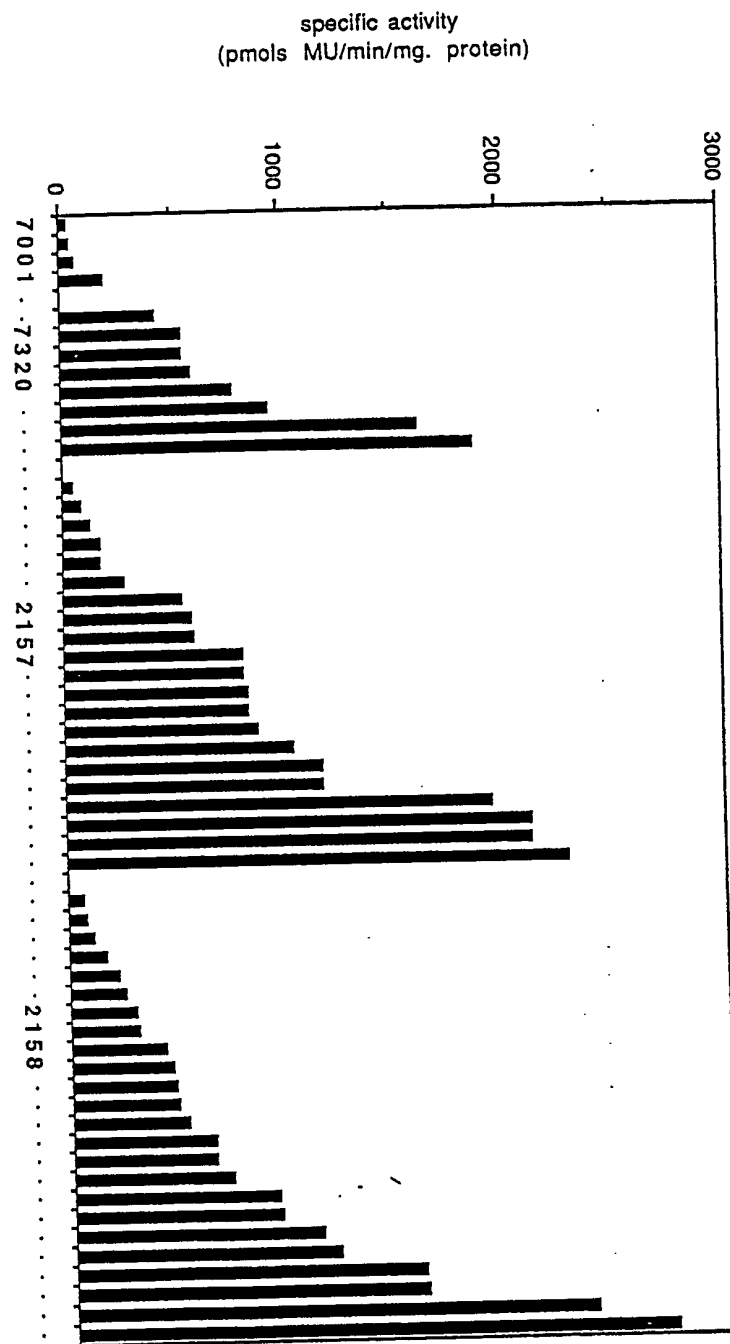
FIGURE 5

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12N 5/00, C12N 1/00, C07H21/04, A01H 1/04

U.S. Cl.: 435/240.4, 317.1; 536/27; 800/1

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷	
Classification System	Classification Symbols
U.S.	435/240.4, 317.1, 320 536/27 800/1

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

USPTO Automated Patent System; DIALOG Files: Biotech, Patents, Agri; Intelligenetics IG Suite Files: Gen Bank, EMBL; See Attachment for Search Terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Gene (The Netherlands), Volume 53, Issued June 1987, Rothstein et al., Promoter cassettes, antibiotic-resistance genes, and vectors for plant transformation", pages 153-161.	1-21
Y	US, A,4,656,131 (KITANO) 7 April 1987, See entire document.	1-21
Y	European Journal of Biochemistry (New York, USA), Volume 155, Issued 1986, Lenstra et al., "Genes coding for the elongation factor EF-1 alpha in Artemia" pages 475-483 (see especially page 481).	1-21

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26 October 1989	19 DEC 1989
International Searching Authority	Signature of Authorized Officer
ISA/US	P.R. Rhodes

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Molecular and Cellular Biology (Washington DC, USA), volume 5, Issued May 1985, Fonzi et al., "Regulation of protein synthesis factor EF-1 alpha in Mucor racemosus", pages 1100-1103.	1-21
Y	Molecular and Cellular Biology (Washington DC, USA), Volume 6, Issued February 1986, Linz et al., "Three Genes for the Elongation Factor EF-1 alpha in Mucor racemosus", pages 593-600.	1-21
Y	Molecular and Cellular Biology (Washington DC, USA), Volume 7, Issued May 1987, Linz et al., "Expression of three genes for elongation factor 1 alpha during morphogensis of Mucor racemosus", pages 1925-1932.	1-21
P Y	Nucleic Acids Research (Oxford, England), Volume 17, Issued June 1989, Pokalsky et al., "Structure and expression of elongation factor 1 alpha in tomato", pages 4661-4673.	1-21

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Form PCT/ISA/210 (extra sheet) (Rev.11-87)

Attachment to Form PCT/ISA/210, Part II.

II. FIELDS SEARCHED SEARCH TERMS:

Elongation Factor, alpha, inventors names.